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AWARD NUMBER: W81XWH-10-1-0023

TITLE: Does RBC Storage Age Effect Inflammation, Immune Function and Susceptibility to Transfusion Associated Microchimerism in Critically Ill Patients? Adverse Effects of RBC Storage in Critically Ill Patients

PRINCIPAL INVESTIGATOR: Philip Spinella, MD

RECIPIENT: Blood Systems Inc. dba Blood Systems Research Institute

REPORT DATE: May 2015

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PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

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W81XWH-10-1-0023

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6. AUTHOR(S)
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Philip J. Norris, MD
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5d. PROJECT NUMBER

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BLOOD SYSTEMS, INC.
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SCOTTSDALE, AZ
85257-1101

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U.S. Army Medical Research
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13. SUPPLEMENTARY NOTES

14. ABSTRACT

Critically ill patients are specifically at risk of adverse effects resulting from the use of RBCs of increased storage age. A large multicenter randomized controlled trial in 30 Canadian centers of 2500 critically ill patients called the Age of Blood Evaluation (ABLE) trial has been completed. In this trial of critically ill patients, which included patients with traumatic injuries, study groups were randomized to either RBCs of < 8 days storage time or standard RBC storage time. The primary outcome of this trial is 90 day mortality. Secondary outcomes include severity of multiple organ dysfunction syndrome, serious thrombotic events and nosocomial infections, and ICU and hospital length of stay. Prospective clinical studies investigating the mechanisms and clinical outcomes associated with increased or decreased RBC storage age in critically ill patients including traumatic injury have not been performed. The ABLE study presents a unique and probably one-time opportunity to investigate mechanisms in the context of clinical outcomes for well-characterized study groups. Our ancillary study was designed to determine specific mechanisms of adverse effects related to the RBC storage age in transfused critically ill patients enrolled in the ABLE study. Specifically we determined if the RBC unit storage time affects patient's immune function, inflammation, coagulation, microparticle concentrations and microchimerism.

15. SUBJECT TERMS
Transfusion, RBC storage age, ICU, clinical trial, extracellular vesicles, coagulation, cytokines, microchimerism

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U	UU	45	19b. TELEPHONE NUMBER *(include area code)*

TABLE OF CONTENTS

1. **INTRODUCTION:** Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.

Our ancillary study is designed to determine specific mechanisms of adverse effects related to the RBC storage age in transfused critically ill patients enrolled in the ABLE study, a large multicenter randomized controlled trial in 30 Canadian centers of 2500 critically ill patients. Specifically we will determine if the RBC unit storage time affects patients' immune function, inflammation, coagulation, microparticle concentrations and microchimerism.

2. **KEYWORDS:**

Transfusion, RBC storage age, ICU, clinical trial, extracellular vesicles, coagulation, cytokines, microchimerism

3. **ACCOMPLISHMENTS:**

Aims

1.) To determine how RBC unit storage time affects inflammation and coagulation in critically ill patients, how these effects change over time after transfusion and if these parameters correlate with clinical outcomes.

1a. Measure the levels of pro-and anti-inflammatory cytokines and coagulation factors in serum from transfused subjects longitudinally using multiplex assays (high and standard sensitivity).

Completion: Cytokine testing has been completed and entered into the database for analysis.

1b. Quantify levels of markers associated with cardiovascular disease including cellular adhesion molecules and growth factors using multiplex bead-based assays.

Completion: Testing of cardiovascular disease markers and growth factors is complete and has been entered into the database for analysis.

1c. Correlate patterns of cytokine and inflammatory marker secretion and measures of coagulation with receipt of blood stored for short vs. long periods.

Completion: Clinical outcomes data has not been acquired yet but will be shortly now that the parent study has been published.

1d. Correlate patterns of cytokine and inflammatory marker secretion and coagulation with all clinical outcomes.

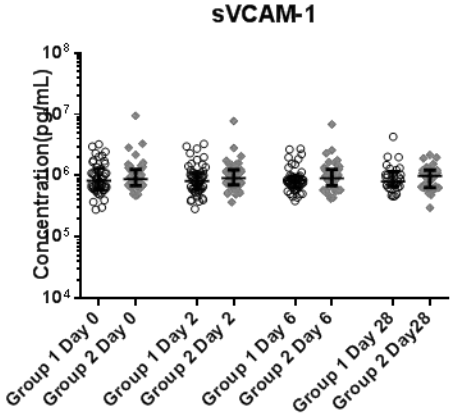
Completion: Clinical outcomes data has not been acquired yet but will be shortly now that the parent study has been published.

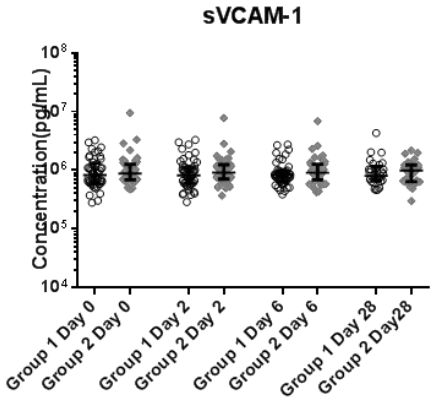
2.) To develop a patient sample repository for future analysis of additional effects of RBC storage age in critically ill patients.

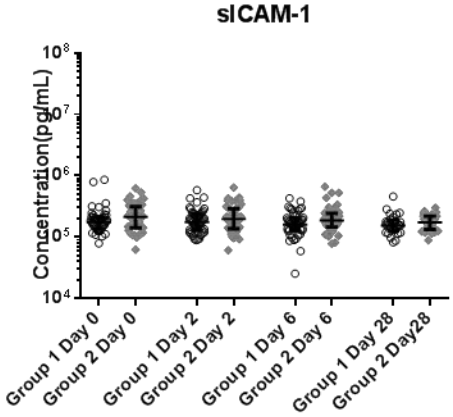
Completion:

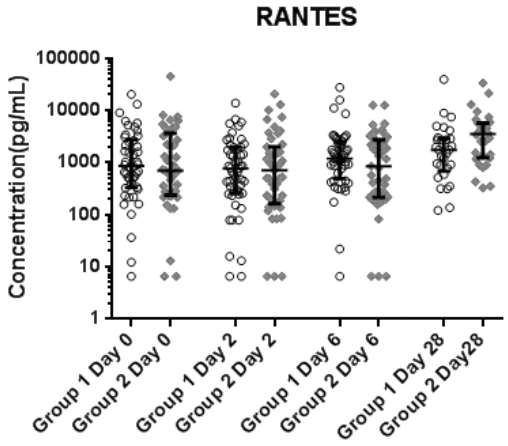
What was accomplished under these goals?

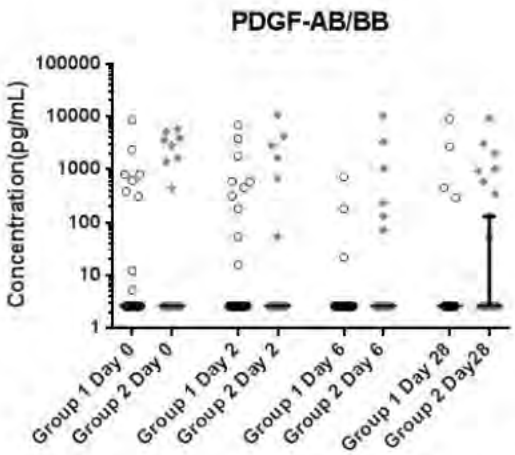
Aim 1: Cytokine, cardiac marker, and growth factor testing: Testing was performed on 100 of 100 planned subjects from the ABLE trial. For the vast majority of analytes, no differences were seen between the two treatment groups (fresh vs. standard issue blood), consistent with the clinical findings of the parent trial. We are still blinded to groups so do not know which group received fresh blood. At day 28 post-transfusion group 2 subjects had marginally higher levels of IL-2 and IFN-γ, though these changes would not be significant after correction for multiple comparisons. Once the clinical data become available the cytokine data will be correlated with outcomes. There is modulation of some cytokines over time, such as IL-6 and IL-8. We anticipate that pairing these data with clinical outcomes will provide insight into the role of inflammation in ICU patient outcomes.







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1000
100
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1
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Group 2 Day 0
Group 1 Day 2
Group 2 Day 2
Group 1 Day 6
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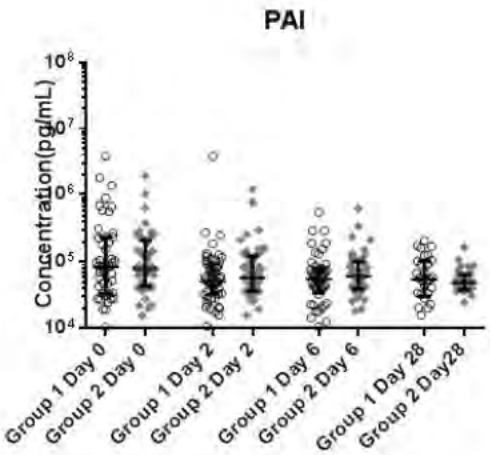
Group 1 Day 28
Group 2 Day28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10^7
10^6
10^5
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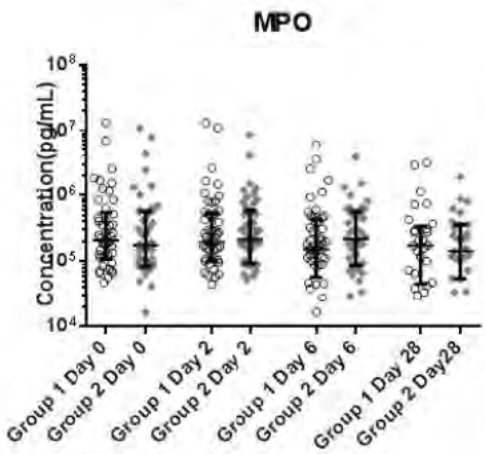
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10^7
10^6
10^5
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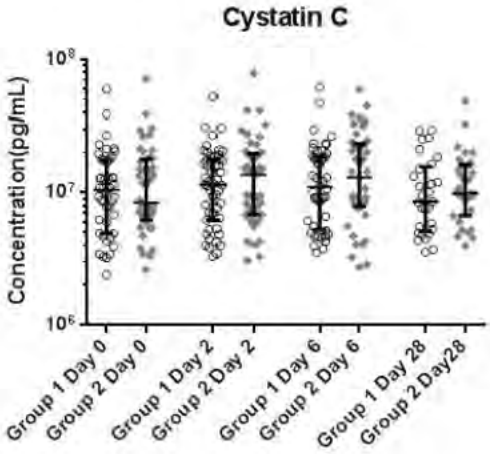
Group 2 Day28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10^7
10^6
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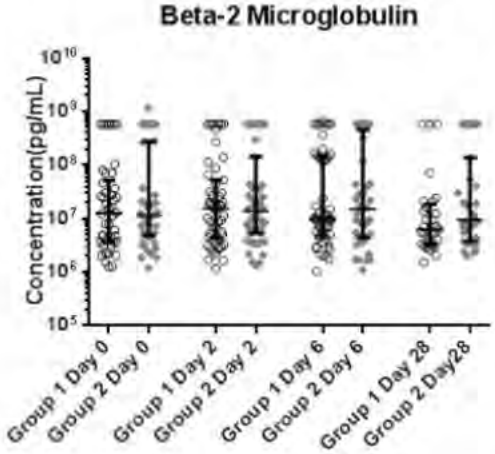
Group 2 Day28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10^9
10^8
10^7
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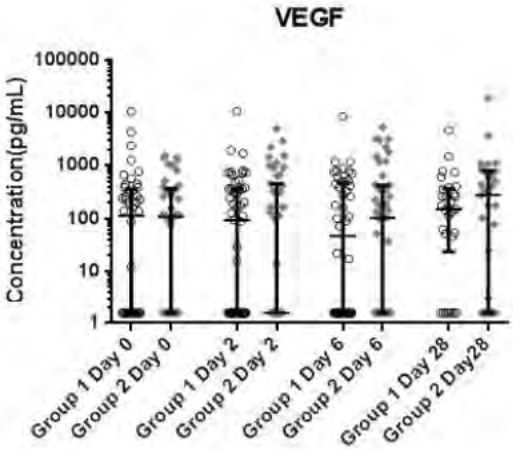
Group 1 Day 6
Group 2 Day 6
Group 1 Day 28
Group 2 Day28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1000
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Group 1 Day 2
Group 2 Day 2
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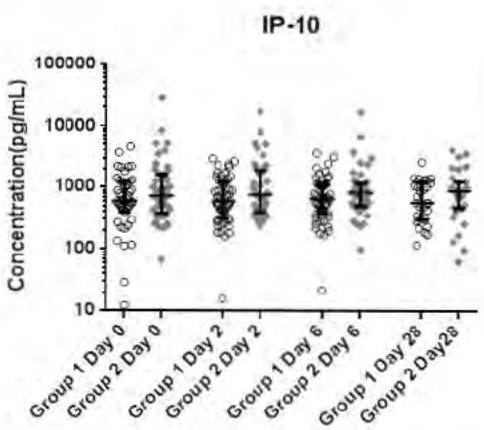
Group 2 Day28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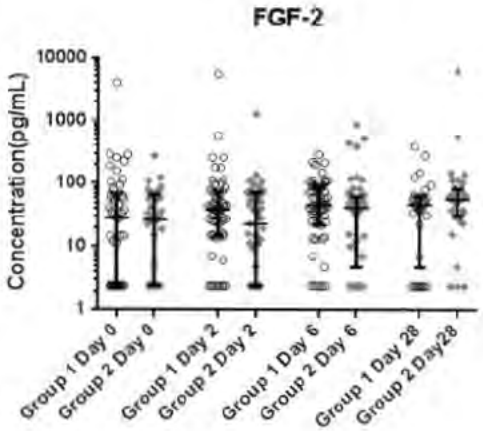
Group 1 Day 28
Group 2 Day28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100
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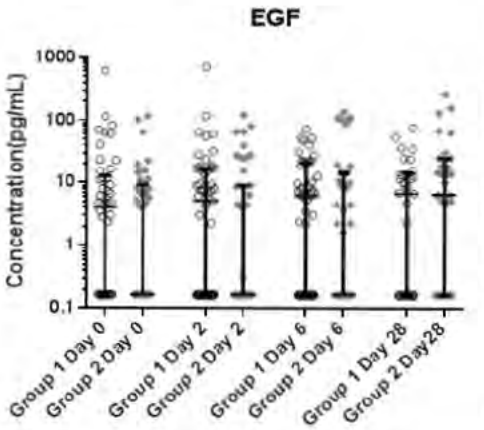
Group 2 Day28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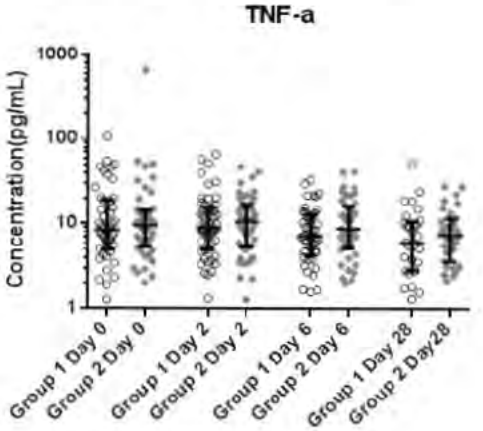
Group 2 Day28


TNF-a
Concentration(pg/mL)
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100
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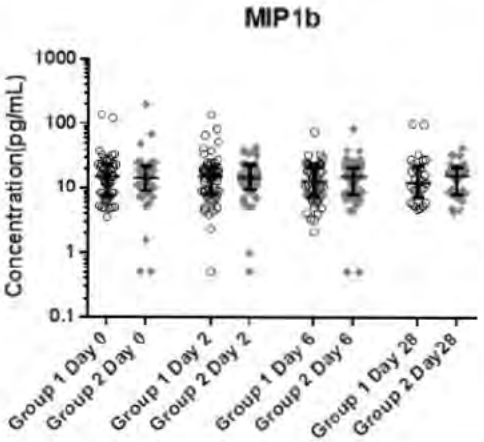
MIP1b
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100
10
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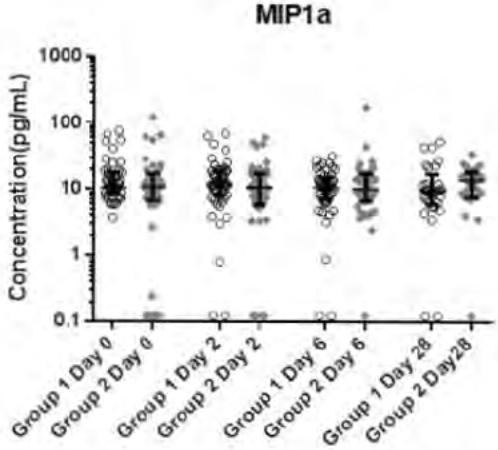
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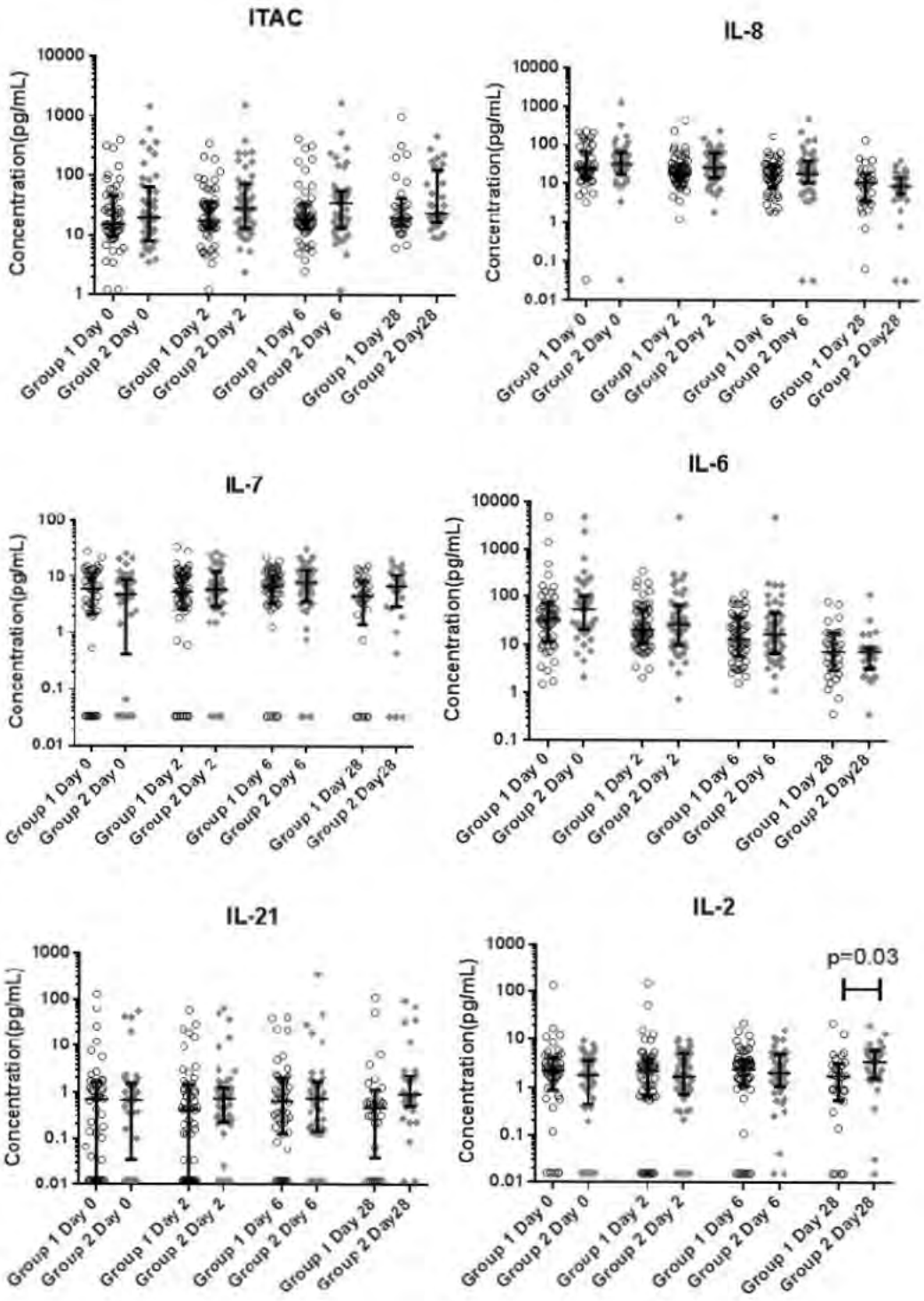
ITAC
IL-8
IL-7
IL-6
IL-21
IL-2
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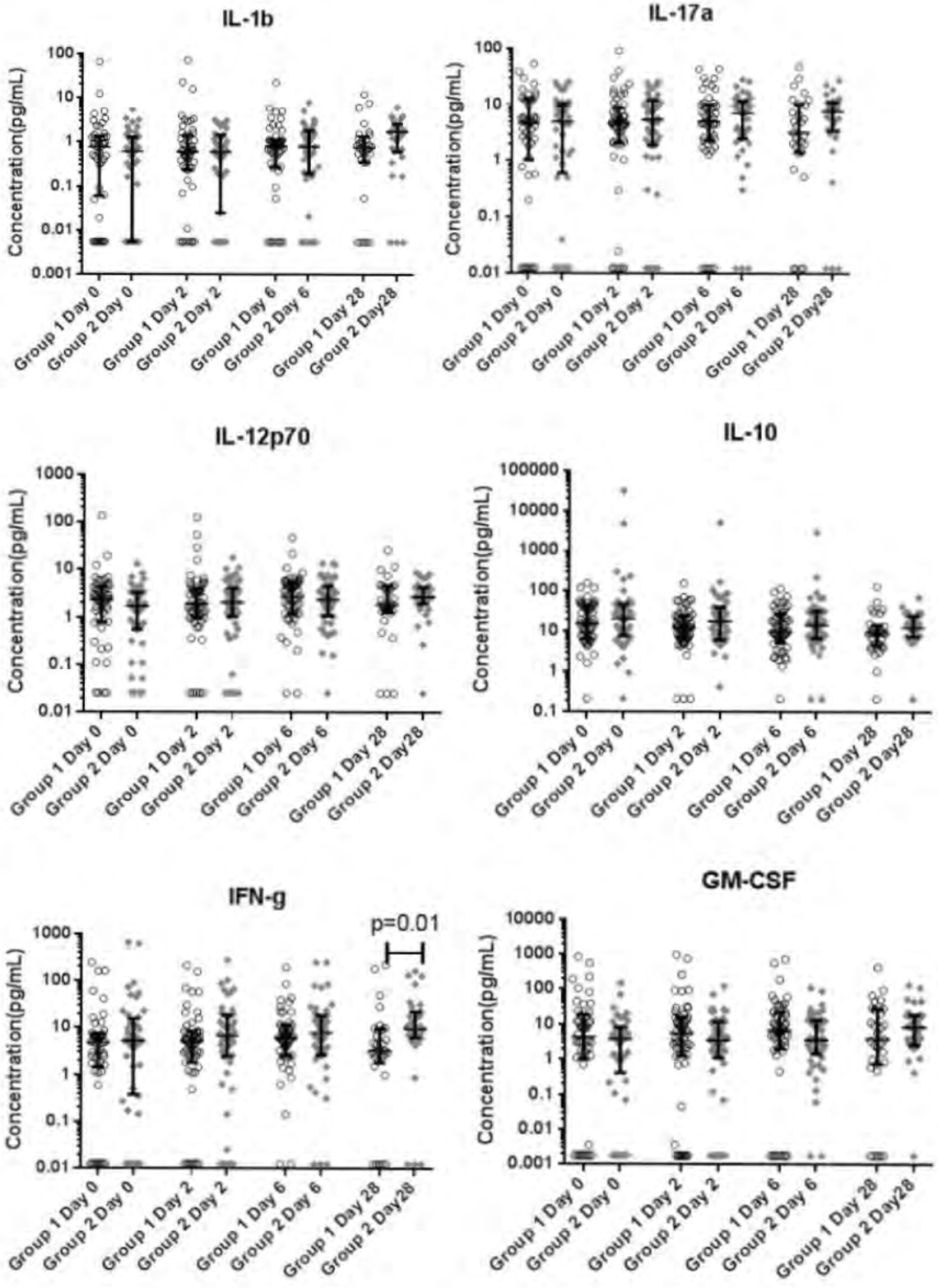
Group 2 Day28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IL-17a
IL-12p70
IL-10
IFN-g
GM-CSF
p=0.01
Concentration(pg/mL)
Group 1 Day 0
Group 2 Day 0
Group 1 Day 2
Group 2 Day 2
Group 1 Day 6
Group 2 Day 6
Group 1 Day 28
Group 2 Day28

What opportunities for training and professional development has the project provided?

Nothing to Report

How were the results disseminated to communities of interest?

To date, two manuscripts have been published (see below).

What do you plan to do during the next reporting period to accomplish the goals?

This is a preliminary (interim) final report. We still have plans to disseminate the results of our studies, as analyses are still being completed. We will present the findings in peer-reviewed publications and at national and international scientific conferences.

4. **IMPACT:** Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:

What was the impact on the development of the principal discipline(s) of the project?

The major premise of the study, that RBCs stored for longer periods would be associated with adverse clinical outcome, was not found to be true. Given that the parent clinical trial did not show a difference between treatment arms, our mechanistic studies of these subjects also did not reveal significant differences between treatment groups. However, the project did generate significant data about coagulation and immunological profiles in critically ill patients. The resulting database will be coupled with the clinical outcome data of the parent trial and will allow analysis of how these parameters correlate with and predict clinical outcome, which will be a significant advance.

What was the impact on other disciplines?

One of the significant by-products of the research was the refinement of techniques to measure sub-cellular fragments, called microparticles or extra-cellular vesicles. In performing the proposed studies under this contract, it was found that spurious levels of microparticles were being detected as positive events. This led to substantial effort to redesign how microparticle testing was performed, and led to the initial two publications in *Cytometry Part A* and the *Journal of Visualized Experimentation*.

Accurate measurement and characterization of microparticles is a rapidly expanding area of research touching multiple disciplines, including oncology, cardiology, and infectious diseases. The techniques we developed are already being translated into transfusion medicine, HIV, and other research areas.

What was the impact on technology transfer?

Nothing to Report

What was the impact on society beyond science and technology?

The results of our research re-affirm the findings of the parent study, namely that fresh blood does not appear to be superior to standard-aged blood in terms of clinical outcomes, or as we have shown in terms of coagulation, microparticle, or microchimerism parameters. While a negative study, this was an important negative to demonstrate, as a positive study would have necessitated substantial (and potentially expensive) changes in how blood is delivered.

5. **CHANGES/PROBLEMS:** The Project Director/Principal Investigator (PD/PI) is reminded that the recipient organization is required to obtain prior written approval from the awarding agency Grants Officer whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:

Changes in approach and reasons for change

Nothing to Report

Actual or anticipated problems or delays and actions or plans to resolve them

The Data Coordinating Center for the parent study has not yet granted access to the clinical data linked to the study subjects. This has delayed our statistical analyses. A final report, complete data analysis, and manuscript preparation are expected in 2015.

Changes that had a significant impact on expenditures

None

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Significant changes in use or care of human subjects

None

Significant changes in use or care of vertebrate animals.

N/A

Significant changes in use of biohazards and/or select agents

None

6. PRODUCTS:

- **Publications, conference papers, and presentations**
  Report only the major publication(s) resulting from the work under this award.

  Journal publications.

1. Inglis HC, Danesh A, Shah A, Lacroix J, Spinella PC, Norris PJ. Techniques to improve detection and analysis of extracellular vesicles using flow cytometry. ***Cytometry A*** (in press).
2. Inglis HC, Norris PJ, Danesh A. Techniques for the analysis of extracellular vesicles using flow cytometry. ***J Vis Exp*** (97) e52484, doi:10.3791/52484 (2015).

- **Technologies or techniques**

As part of this program our group made significant advances in how to quantitate and characterize extracellular vesicles. These data have been published in peer-reviewed journals.

Inventions, patent applications, and/or licenses

None

Other Products
None

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

Name:	Philip Spinella, MD
Project Role:	Principal Investigator
Researcher Identifier (e.g. ORCID ID):	Unknown
Nearest person month worked:	.6
Contribution to Project:	Dr. Spinella served as the Principal Investigator of the project, responsible for overseeing all research-related activities and reporting.
Funding Support:	

Name:	Philip Norris, MD
Project Role:	Co-Principal Investigator
Researcher Identifier (e.g. ORCID ID):	0000-0003-0526-2088
Nearest person month worked:	.07
Contribution to Project:	Dr. Norris served as Co-PI of the project and oversaw cytokine testing.
Funding Support:	N/A

Name:	Avani Shah
Project Role:	Project Coordinator
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	2
Contribution to Project:	Ms. Shah interacted with the clinical sites to ensure enrollment of ABLE subjects in the current substudy. She also interacted with staff at BSRI to ensure processing of samples and generation of the sample repository.
Funding Support:	N/A

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to Report

What other organizations were involved as partners?

1. The Washington University; St. Louis, MO; Collaborator
2. Ottawa Hospital Research Institute the research arm of The Ottawa Hospital; Ottawa, Canada; Clinical Site
3. Institit Universitaire de Cardiologie et de Pneumologie, Hôpital Laval; Quebec, Canada; Clinical Site
4. CHU de Quebec; Quebec, Canada; Clinical Site
5. St. Michael's Hospital; Ontario, Canada; Clinical Site
6. Capital District Health Authority; Halifax, Nova Scotia; Clinical Site

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: For collaborative awards, independent reports are required from BOTH the Initiating PI and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to **https://ers.amedd.army.mil** for each unique award.

9. APPENDICES

Cytometry
PART A
Journal of the International Society for Advancement of Cytometry

MIFlowCyt

Techniques to Improve Detection and Analysis of Extracellular Vesicles Using Flow Cytometry

Heather C. Inglis,[1] Ali Danesh,[1,2] Avani Shah,[1] Jacques Lacroix,[4] Philip C. Spinella,[5] Philip J. Norris[1,2,3]*

[1]Blood Systems Research Institute, San Francisco, California

[2]Department of Laboratory Medicine, University of California, San Francisco, Califoria

[3]Department of Medicine, University of California, San Francisco, California

[4]Division of Critical Care, Department of Pediatrics, University of Montreal, Quebec, Canada

[5]Division of Critical Care, Department of Pediatrics, Washington University School of Medicine, St. Louis, Missouri

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Grant sponsor: NIH, Grant numbers: HL095470; U01 HL072268

Grant sponsor: DoD contracts, Grant numbers: W81XWH 10 1 0023; W81XWH 2 0028

Additional Supporting Information may be found in the online version of this article.

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E mail: pnorris@bloodsystems.org

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DOI: 10.1002/cyto.a.22649

Abstract

Extracellular vesicles (EVs) range in size from 50 nm to 1 μm. Flow cytometry (FCM) is the most commonly used method for analyzing EVs; however, accurate characterization of EVs remains challenging due to their small size and lack of discrete positive populations. Here we report the use of optimization techniques that are especially well suited for analyzing EVs from a high volume of clinical samples. Utilizing a two pronged approach that included 1) pre filtration of antibodies to remove aggregates, followed by 2) detergent lysis of a replicate sample to account for remaining false positive events, we were able to effectively limit false positive non EV events. In addition, we show that lysed samples are a useful alternative to isotypes for setting gates to exclude background fluorescence. To reduce background, we developed an approach using filters to "wash" samples post staining thus providing a faster alternative to ultracentrifugation and sucrose gradient fractionation. In conclusion, use of these optimized techniques enhances the accuracy and efficiency of EV detection using FCM. © 2015 International Society for Advancement of Cytometry

Key terms

microparticles; flow cytometry; antibody aggregates; filtration; extracellular vesicles

Statement of Purpose

Extracellular vesicles (EVs) are small, submicron sized vesicles released from multiple cell types (1) that play an important role in intercellular communication (2). EVs range in size from 50 nm to 1 μm, roughly 1/200th to 1/10th the size of an average human cell (3) and can be detected in bodily fluids such as blood, urine, semen, and saliva (2). EVs can be further categorized into smaller groups of cell derived vesicles such as exosomes and apoptotic bodies on the basis of their size and mechanism of formation (4). The classification and nomenclature of cell derived vesicles continues to be a topic of ongoing debate (4,5); however, here we will use the general term "EVs" to refer to all types of extracellular biological vesicles released by cells.

Though once considered to be uninteresting debris and discounted as mere artifact, EVs are increasingly being recognized as ubiquitous, key players in the body's complex network of intercellular signaling (6,7). The rate of EV release, while constitutive in healthy cells, is greatly increased when cells are subjected to stress, activation, stimulation, or disease (7 9). EVs express different surface markers depending on their cell of origin and immune or coagulation activation status (2,10,11). Expression of these markers enables EVs to act as physiological signal mediators, playing either immunosuppressive or immunostimulatory roles in cell cell communication (12,13) in addition to affecting coagulation and vasoregulation (14). Some circulat

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International Society for Advancement of Cytometry

ing EVs have been shown to promote tumor progression by suppressing immune function (15 19) and supporting tumor cell migration in metastasis (20 23), while others have been shown to suppress disease progression by conferring therapeu tic benefit in the treatment of diseases such as ischemia (24) and kidney disease (25). Clinically, EVs have a wide range of applications in diagnostics and disease therapy. EVs in biolog ical fluids can be monitored for disease biomarkers, as con centrations of some EVs are known to be associated with increased risk of specific diseases and cancers, including lym phoma (26), lung (27), breast, gastric (28), colorectal, pros tate, kidney, and ovarian cancer (29 33) and cardiovascular disease (34). In addition to their use in disease monitoring, some researchers are utilizing EVs to develop new treatments and anticancer therapies. The roles of certain EV subtypes to inhibit tumor growth has been investigated as a potential treatment for cancer (35,36). More recently, researchers have shown that EVs can be manipulated to deliver tailored thera peutic cargo to specific targets within the body (37,38).

A number of methods have been used to analyze EVs, including scanning electron microscopy (39,40), transmission electron microscopy (TEM) (41), atomic force microscopy and dynamic light scattering (42 45), and western blotting (46,47). Clinically, flow cytometry (FCM) is the most com monly used method for analyzing EVs in blood (5,48 50); however, accurate characterization of EVs remains challeng ing. Perhaps some of the most significant problems associated with measuring EVs using FCM stem from the ability/sensitiv ity of this method to properly discriminate positive from neg ative events. This is due mainly to the small size of the EVs, which results in (1) less fluorescence emitted due to the fewer number of antigens per particle and (2) limited feasibility of post stain washing to reduce background fluorescence. Fur thermore, because FCM uses a triggering threshold to initiate a signal, electronic noise and particulates in the sheath and sample buffer can generate very high background signals, which can drown out/overwhelm very small signals created by EVs. Using a side scatter threshold, researchers have reported being able to differentiate 100 nm from 300 nm beads (48), however, EVs have a lower refractive index than beads which limits their detection at these lower limits. Some researchers prefer to use a fluorescent channel as the triggering threshold (51), however, this is complicated by the fact that no pan specific marker for all EVs exists. Annexin V, once considered to be a robust EV marker by binding to EV surface phosphati dylserine (PS), has more recently been shown to fail to bind to the majority of EVs (52,53), with binding being greatly affected by calcium concentrations and pre analytical condi tions (54). Therefore, while specific subpopulations may be better detected using a fluorescence threshold, all other subpo pulations (those not carrying the fluorescent marker) will not be detected using this method. When analyzing very small particles, accuracy depends on the proper discrimination of EVs from other non cell derived particles and on thorough removal of background noise. Prior publications have noted difficulties associated with using FCM to analyze EVs, includ ing false positive signals arising from EV mimicking immune complexes (55,56), self aggregation of antibodies due to agita tion (57), and limited applicability of traditionally used FCM controls such as FMO (fluorescence minus one) and/or anti body isotypes (58). EV sample collection and processing is yet another area in which standardization is needed, yet no con sensus exists on an optimal protocol (59 61). Many different pre analytical variables have been shown to affect EV content, including storage temperature and duration (62,63), anticoa gulant/preservative used (62,64), and centrifugation method used (59,63). Specialized techniques and optimized protocols have been recommended (61); however, there is no consensus on the best method for EV detection by FCM.

Our laboratory is performing flow cytometric analyses of the circulating EV concentration and phenotype in critically ill patients transfused with blood stored for short vs. standard storage periods. Faced with a high volume of samples to test, we needed to refine our protocol in a way that would both minimize processing time and maximize accuracy and effi ciency. Here, we present the results of EV optimization studies that were performed on healthy controls to ensure the accu racy and efficiency of EV analysis before quantifying EVs in plasma from study subjects. Our optimization experiments focused on four key areas: removal of aggregated fluorochrome conjugated antibodies prior to EV staining, washing of EVs after staining, the optimal control sample to use as the basis of setting gates to count positive EV events, and the effect of EV concentration on EV quantitation and the proportion of positive events measured.

Materials and Methods

Study Subjects

Study samples were obtained from subjects in the Age of Blood Evaluation (ABLE) trial (65). Intensive care unit (ICU) patients were randomly assigned to receive either fresh (< 7 days' storage) or standard (expected mean ≈ 21 days' storage) blood for transfusion. Whole blood was collected from these patients on Day 0 (before transfusion) and on Days 2, 6, and 28 post transfusion. Some optimization steps were performed using samples from discarded Trima leukoreduction system chambers (LRSCs) from Blood Centers of the Pacific or from whole blood collected from six healthy volunteers in citrate tubes. All human subject samples were tested under an institu tional review board (IRB) approved protocol and with informed consent of the subjects.

EV and Cell Processing

EVs were isolated from whole blood using a common dif ferential centrifugation technique described in the literature (59,66 68). Immediately after collection, tubes were centri fuged at 1,500*g* for 10 min to separate cells from the superna tant, then at 13,000*g* for 10 min to remove platelets. The supernatant was carefully removed, and this platelet poor plasma (PPP) was used to study EV concentration and pheno type. PPP from six normal donors was combined to create a normal donor pool. Aliquots of 0.5 mL were stored at 80°C (refer to Fig. 1A for overview). Some optimization steps used EVs which had been concentrated using an additional

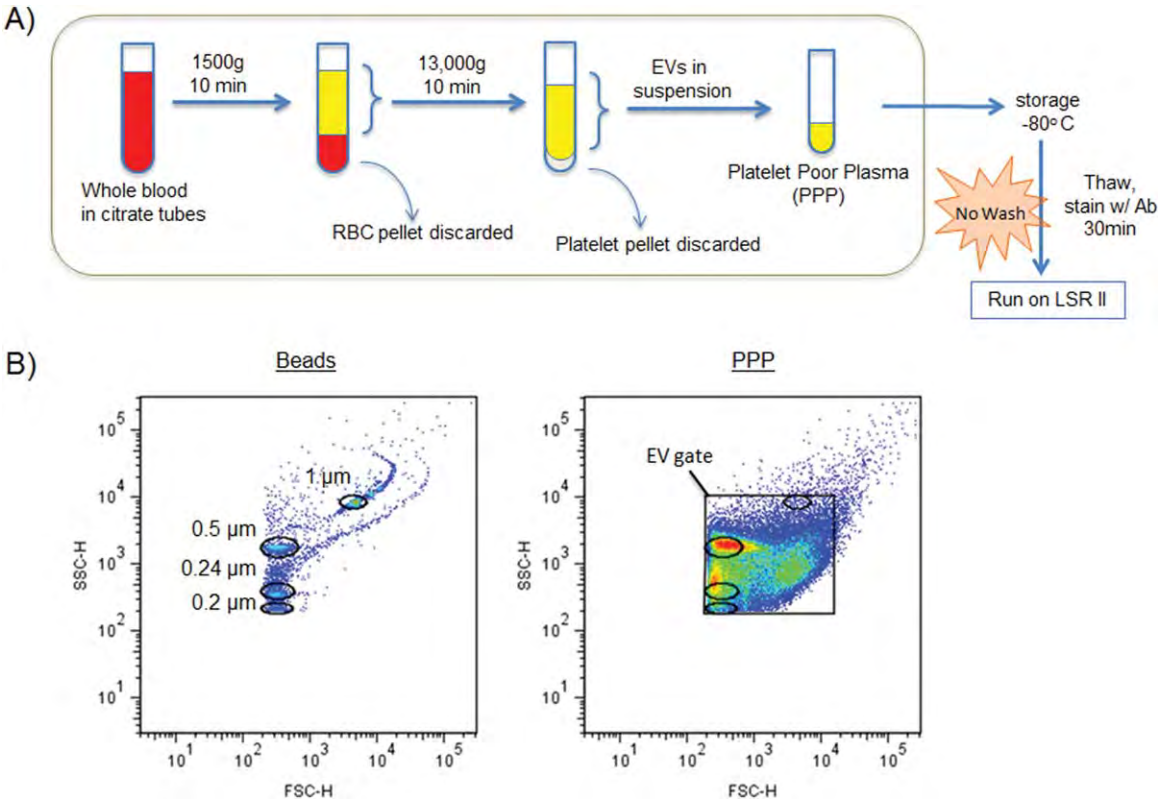

Figure 1. Processing scheme and FCM setup. **A**: Initial method for isolation, storage, and analysis of EVs. Note: post optimization protocol includes pre stain antibody filtration and post stain EV filtration steps. PPP platelet poor plasma. **B**: Biparameter FSC H vs. SSC H dot plot showing locations of 0.2, 0.24, 0.5, and 1 µm sizing beads used to set up FSC and SSC voltages for FCM analysis. PBS alone (plot not shown because no events detected by cytometer) was also used to determine the maximum voltages able to exclude the majority of elec tronic noise. Right plot shows a PPP sample with the EV gate drawn and bead region gates overlaid. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

centrifugation or filtration step. In these instances, the EVs are referred to as "concentrated" in figure legends. When centrifu gation was used to concentrate EVs, 3 mL of PPP was added to 32 mL phosphate buffered saline (PBS, UCSF Cell Culture Facility, San Francisco, CA) and spun for 60 min at 100,000*g*. EV pellets were re suspended in 1 mL RPMI 1640 (Invitro gen, Carlsbad, CA) and stored at 80°C. When filtration was used to concentrate EVs, 1.5 mL of PPP was added to centrif ugal filters and resuspended in 400 µL PBS for immediate analysis (see below for full filtration method protocol). Peripheral blood mononuclear cells (PBMCs) were isolated from Trima LRSCs on a Ficoll Paque PLUS density gradient (GE Healthcare Bio Sciences, Piscataway, NJ). Aliquots of 20×10^6 cells were frozen in media that contained 90% fetal bovine serum (HyClone, Logan, UT) and 10% dimethyl sulf oxide (Fisher Bio Reagents, Pittsburgh, PA) and stored in liq uid nitrogen vapor.

Antibodies

In order to examine EVs for cell of origin markers (phe notype) and immune or coagulation activation markers, we used several different fluorochrome conjugated monoclonal antibodies, including red blood cell markers: CD108 PE and CD235a FITC, a platelet marker: CD41a PerCP/Cy5.5, and leukocyte markers: CD3 PerCP/Cy5.5, CD19 Alexa700, CD28 FITC, CD16 V450, CD152 APC, CD14 APC/Cy7, and CD62 L APC. All isotype controls were matched to their respective antibodies according to their fluorochrome type, concentration, heavy chain (IgA, IgG, IgD, IgE, or IgM), sub class, and light chain class (kappa, lambda). Refer to Table 1 for a detailed summary of the antibodies that were used in the experiments described in this article.

Antibody Labeling

PPP samples were rapidly thawed and 50 or 100 µL were added to 2 5 µL of each monoclonal antibody. Prior to testing EV samples, each antibody was titrated using serial dilutions to determine the "saturating" concentration (the lowest con centration which yielded nearly maximal fluorescence). Sam ples were incubated at 4°C for 30 min and either filtered or re suspended in 400 µL PBS for immediate FCM analysis.

Absolute Count Analysis

Trucount™ tubes (BD Biosciences, San Diego, CA) with a known number of fluorescent beads were utilized for EV quantification. To each Trucount tube, 50 µL sample and 350 µL PBS were added and samples were read immediately on the flow cytometer. EV concentrations were calculated using the following equation:

Table 1. Antibody characteristics

MARKER	FLUOROCHROME	MFGR	CAT#	CLONE	ISOTYPE
CD3	PerCP Cy5.5	Biolegend	344808	sk7	Ms IgG1, κ
CD14	APC Cy7	Biolegend	301820	M5E2	Ms IgG2a, κ
CD16	V450	BD	560474	3G8	Ms IgG1, κ
CD28	FITC	Biolegend	302906	CD28.2	Ms IgG1, κ
CD152	APC	BD	555855	BNI3	Ms IgG2a, κ
CD19	A700	Biolegend	302226	HIB19	Ms IgG1, κ
CD41a	PerCP Cy5.5	BD	340930	HIP8	Ms IgG_1, κ
CD108	PE	BD	552830	KS 2	Ms IgG2a, κ
CD62L	APC	Biolegend	304810	Dreg56	Ms IgG1, κ
CD235a	FITC	Biolegend	349104	GA R2 (HIR2)	Ms IgG2a, κ

$$\text{EVs}/\mu\text{L} = [\text{EV region events}/\text{bead region events}] \times [\text{Trucount}^{\text{TM}} \text{ beads}/\mu\text{L of sample added}] \quad (1)$$

Data Collection

Acquisition was performed using a 3 laser (20 mW Coher ent Sapphire 488 nm blue, 25 mW Coherent Vioflame 405 nm violet, and 17 mW JDS Uniphase HeNe 633 nm red) LSR II benchtop flow cytometer equipped with FACS Diva 6.0 software (BD Biosciences). Specific cytometer parameters and filter con figurations used for acquisition are outlined in Table 2. Flow cytometer setup was performed using CS&T instrument setup beads (BD Biosciences). Forward scatter (FSC) and side scatter (SSC) parameters were set to log mode and the lowest threshold allowed by the cytometer (200) was selected for each. Compen sation setup was performed using AbC beads (Invitrogen) and compensation values were determined by FACS Diva software. FSC/SSC voltages were set to the highest values that excluded the majority of background noise (i.e., just below the voltage threshold at which event rate surpassed 5 events/sec while run ning a tube of PBS alone). Typically, this threshold occurred at FSC and SSC voltages of around 500 600 and 300 350, respec tively. Rainbow fluorescent particles (Spherotech, Lake Forest, IL) were used to adjust all channel voltages between batches in order to maintain voltage consistency from run to run. Figure 1B shows the location of the EV gate in relation to 0.2, 0.24, 0.5, and 1 μm beads (0.5 mL of Megamix Plus SSC; BioCytex, Mar seille, France) beads combined with 1 2 drops of Spherotech Ultra Rainbow Fluorescent Particles). These beads cannot be used to determine EV size but are useful for showing the rela tive sizes of EVs detected. All samples were acquired at low sam ple pressure and low flow rate (~8 12 μL/min). In pre optimization experiments (Fig. 2 and Supporting Information Figs. 1 and 2), collection of 100,000 events was attempted for each sample, and in situations with very few events, tubes were run for at least 3 min. Post optimization, each sample (includ ing lysed samples) was run for exactly 1 or 2 min to allow for the subtraction of false positive events detected in the lysed sam ple over an equal time frame. FCS files were exported and data were evaluated using FlowJo software (TreeStar, Ashland, OR; Mac version 9.6.1 or PC version 7.6.5).

Transmission Electron Microscopy

TEM was used to visualize antibodies using negative staining. Approximately 10 μL of each sample was added to a Formvar coated 300 mesh copper grid and allowed to adhere for 2 min at room temperature. Excess liquid was removed by blotting the edge of the grid with filter article. Next, a drop of 2% aqueous uranyl acetate solution was applied to the grid for 30 sec. The excess stain was removed as before and the speci mens were examined by TEM using a JEOL JEM 1400 elec tron microscope.

Lysis Step

In order to discriminate between EVs and protein aggre gates, we used a lysis technique similar to that described by György et al. (55) to reveal false positive events. A non ionic detergent, 10% Nonidet P 40 (NP 40) (New England Biolabs, Ipswich, MA) was used to lyse EVs. After an initial reading on the flow cytometer, stained EV samples were added to 20 μL of 10% NP 40 (final concentration 0.5% NP 40). Samples were then re read and compared to the initial reading. Antibody positive events remaining in the lysed sample were subtracted from the positive events in the initial reading to determine the proportion of true EVs.

Filtration

Antibody filtration was performed prior to staining using Ultrafree® MC/Durapore® PVDF centrifugal filter units of various pore sizes (0.1, 0.22, 0.45, and 0.65 μm, Millipore, Bedford, MA). For each panel, titrated antibodies (2 5 μL each) were combined and added to the top of a filter, the tube was centrifuged in a fixed angle single speed microcentrifuge (Fisher Scientific, Pittsburgh, PA) for ~ 30 sec, and the filtrate was used for staining. Post stain filtration was performed using the same filters and same filtration process, however, the filtrate was discarded and EVs remaining on the filter surface were resuspended in 400 μL PBS and saved for flow analysis. Originally, post stain filtration was performed at 600*g* for 30 sec. After testing a variety of PPP samples, it was ultimately decided that an increase to 800*g* for 2 5 min would be neces sary to accommodate all PPP samples, some of which required a slightly higher force to move through the filter effectively.

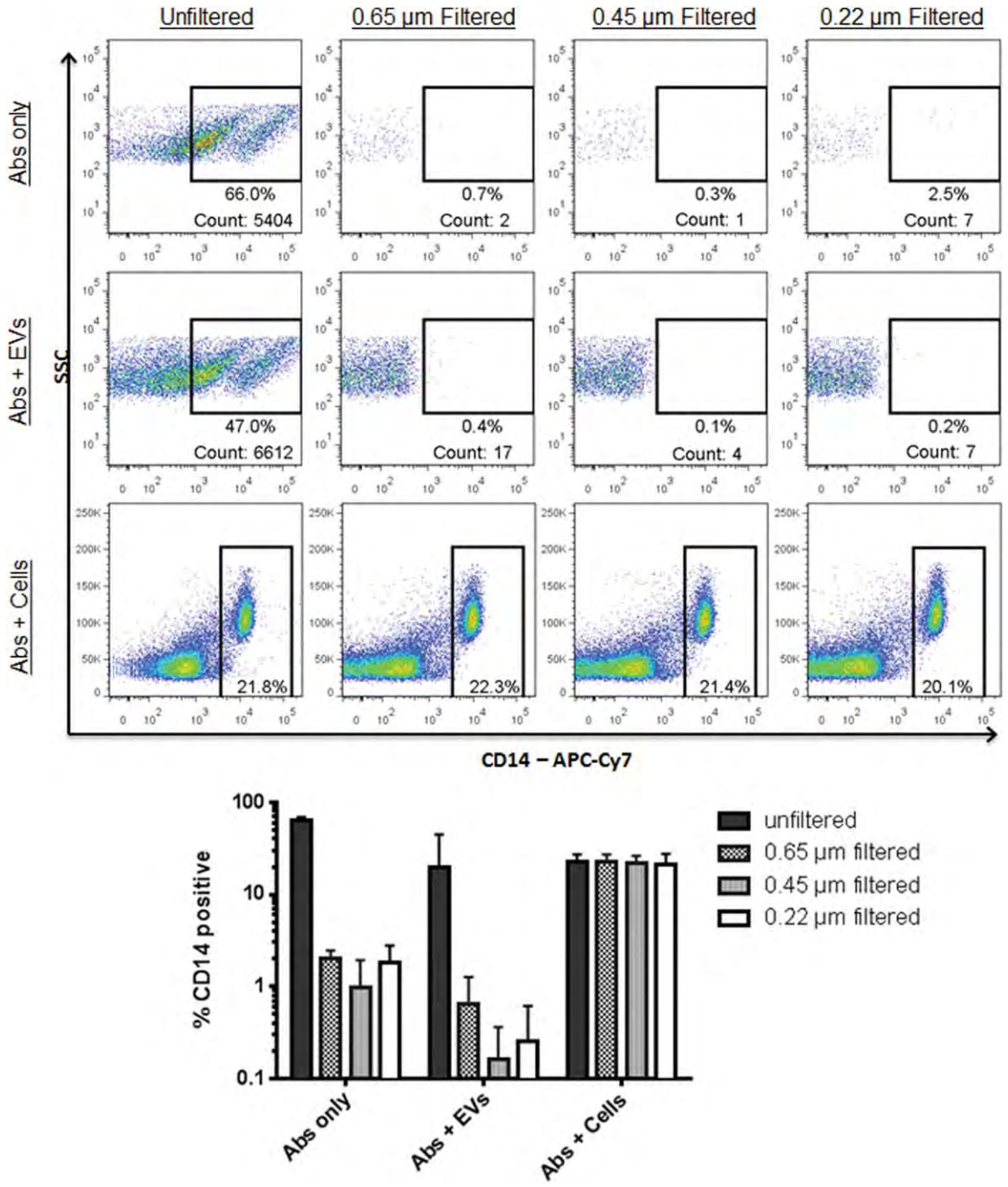

Figure 2. Effectiveness of filtering and centrifuging to remove antibody aggregates. Unfiltered or filtered antibodies were added to PPP or PBMCs, and the percentage of CD14 positive events was determined. Events shown in the top two rows are within the FSC/SSC EV gate. Events shown in the bottom row are within the FSC/SSC lymphocyte gate. The bar graph shows the summary data from three replicate experiments. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Statistical Analysis

Nonlinear regression analysis using a semi log line was used to assess the correlation between EV concentration and percentages of antibody positive events. The two way ANOVA test followed by Tukey's multiple comparisons test was used to evaluate differences in the percentages of $CD14^+$ events after different sized filters were used to remove antibody aggregates in EVs and PBMCs. For EV dilution experiments, slopes were determined by nonlinear regression analysis using a log log line, and *R* squared values were determined by nonlinear regression analysis using a log log line and slope constraint equal to 1.0 in order to assess goodness of fit. The *t* test corrected for multiple comparisons using the Holm Sidak method was used for comparing the

Table 2. LSR II configuration

LASER	DETECTOR	LP MIRROR	BP FILTER	FLUOROCHROME	MARKER	ALT. MARKER
Blue	A	735	780/60	PE Cy7		
	B	685	695/20	PerCP Cy5.5	CD3	CD41a
	C	635	670/14			
	D	552	575/26	PE	CD108	
	E	505	530/30	FITC	CD28	CD235a
	F		488/10	SSC		
Violet	A	505	525/50			
	B		440/40	V450	CD16	
Red	A	735	780/60	APC Cy7	CD14	
	B	685	720/45	Alexa 700	CD19	
	C		660/20	APC	CD152	CD62L

LP long wavelength pass, BP band pass.

numbers of antibody positive events remaining after filtration or centrifugation was used to remove antibody aggregates. The two tailed *t* test for paired comparisons was used to compare the mean percentages of antibody positive events before and after post stain filtration. Statistical significance was defined as $P < 0.05$. All statistical analyses were performed using Prism 5 (GraphPad Software, La Jolla, CA).

Results and Discussion

Antibody Aggregates Fall Within the EV FSC/SSC Gate

PPP was isolated from clinical study or normal donor patient samples using a differential centrifugation process commonly found in the literature (59,66 68) (Fig. 1A). Some of the larger EVs may have been removed with the relatively long duration centrifugation that we used. The general techniques we describe in this article, however, are applicable to all FCM analysis of EVs and not specific to EVs derived from PPP. Samples were then stained with one or more antibody conjugated fluorochromes. When ABLE study samples were tested, which varied widely in EV concentration (EV/μL), a negative correlation between EV concentration and the proportion positive for cellular markers was found. The samples taking the longest to acquire 100,000 events on the cytometer, i.e., samples having the lowest EV concentrations, had the highest background and thus the highest number of positive events. Supporting Information Figure 1A shows the results of EV analysis of two representative donors, one with a low concentration of EVs (top row) and one with a high concentration (bottom row). Plotting results from all donors revealed a significant negative correlation between EV concentration and positive events for four markers (Supporting Information Fig. 1B). To test whether the positive events could be due to artifact, antibody alone in PBS (without EVs added) was tested, and the same pattern of positive events was observed as in the samples having low EV concentration (Supporting Information Fig. 1C). Since the antibody without EV sample showed more signal than the antibody with EV, this suggested that the signal was artifactual.

Eliminating Antibody Aggregates

To eliminate false positive events, the efficacy of filters to remove presumed antibody aggregates from the EV gate was tested. We experimented using several different sized filters (0.1, 0.22, 0.45, and 0.65 μm) and found that all filters were highly effective at removing aggregates without compromising the antibody's ability to stain PBMCs (Fig. 2). Next, to determine the most suitable method for removing antibody aggregates, we compared our filtration method against a common centrifugation method found in the literature (17,000*g* for 5 min) (69). Filtration was more effective than centrifugation at removing aggregates from all antibodies tested, and this was confirmed by electron microscopy (Supporting Information Fig. 2). Of note, longer centrifugation times than we used in the current experiments have been described (69), but we only tested a 5 min centrifugation in the current work in an attempt to develop a protocol suited to high throughput sample analysis. Similar 5 min centrifugation protocols to remove antibody aggregates were recommended by other researchers as well, including 16,000*g* for 5 min (70) and 18,000*g* for 5 min (63). Antibodies filtered with smaller pore sizes were equally as effective as those filtered with larger pore sizes. We did not experience problems with clogging of the filters using any pore size during antibody filtration. One drawback to using filters for antibody aggregate removal is the cost; the retail price of each filter is ~$2. However, because all antibodies can be combined in one tube before filtering, if 40 samples were run per batch, the cost would be 5 cents per sample. In batched analyses, the filter for removing antibody aggregates comprises a small fraction of the total cost of testing each sample.

EV Gating Strategies

The final step in successfully analyzing FCM data is setting gates to separate positive from negative events. Many researchers use isotypes to do this (60,71,72), though a lysis method for identifying false positive EV events has been described (55,56). The lysis step utilizes a detergent to disrupt EVs, with immune complexes and other non EV related events remaining after detergent lysis eliminates the EVs. In

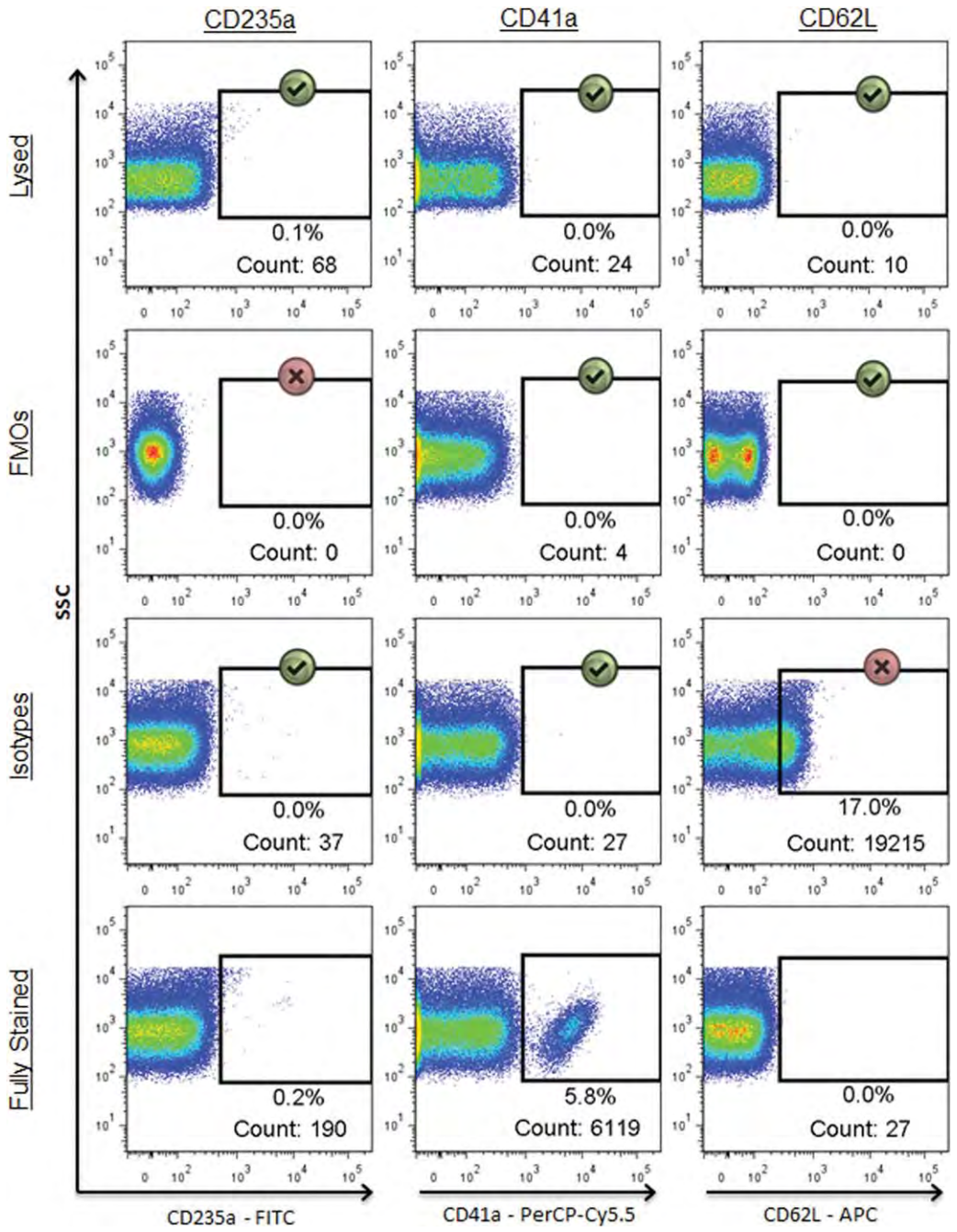

Figure 3. Detergent lysis assists in setting gates for positive events. Events shown are within the FSC/SCC EV gate. Comparison of three different negative controls (FMO, isotype, or lysed) in their ability to provide appropriate indications of background fluorescence across three different markers in a fully stained sample (bottom row). Gates for each marker were made using the lysed sample (top row) and then copied to the rows beneath. Green check marks indicate instances in which the background fluorescence appropriately matches that of the corresponding marker in the fully stained sample, while the red X's denote controls which poorly predicted the background fluorescence in the fully stained sample.

samples with paired lysed and unlysed samples, the lack of a true EV population expressing CD16 can be appreciated (Supporting Information Fig. 3A). After optimizing the use of a lysis step, lysed samples were compared to isotype stained samples for setting background fluorescence gates (Supporting Information Fig. 3B). In these examples the donor's lysed sample was superior to the isotype control for defining background fluorescence for the CD235a and CD41a antibodies because the background more closely matched that of the fully stained Ab sample. Similarly, Figure 3 shows the ability of three different negative controls (FMO, isotypes, and lysed) to accurately predict the background fluorescence of a fully stained, non post stain filtered sample. FMO controls provided an appropriate indication of background fluorescence

for CD41a and CD62L but not for CD235a stains. Isotype controls accurately portrayed background fluorescence in CD235a and CD41a but failed to do so for CD62L. Background fluorescence of the lysed sample, on the other hand, matched all three markers in the fully stained sample. After analyzing a large panel of samples in a similar fashion to Figure 3, lysed controls worked as well as isotype controls for setting background fluorescence gates 72% of the time (83/116) and better than isotype controls in 28% of cases examined (33/116). These results demonstrate that the selection of an appropriate control sample to use for gating can vary depending on the antibody and marker measured.

Currently, many researchers using FCM to analyze EVs use isotype controls as a means for setting positive vs. negative discrimination gates (55,56,60,71,72). However, our attempts to replicate the background of an antibody with its isotype proved to be difficult, as it was impossible to know whether the signal was true background or simply an artifact caused by the differences in spectral properties between the two stains. Indeed, a number of publications have noted similar issues associated with using isotype controls for this purpose (58,59). Isotype gates can vary widely depending on a number of factors including: antibody supplier, fluorochrome:protein ratio, antibody concentration, propensity for aggregation, and antibody subclass (7,48,58). Though these variables can be accounted for/controlled to a certain degree, it is difficult if not impossible to match perfectly the background fluorescence of a fluorochrome conjugated antibody to that of its isotype. Considering the low number of antigens per EV (and correspondingly small fluorescence signal emitted), even minute differences in background signal between an antibody and its isotype will significantly affect proper gate placement and the ability to accurately distinguish positive events. Of all of the controls we tested (FMO, unstained, isotype, and lysed), lysed samples proved to be the most consistently reliable as an indicator of background fluorescence across all markers when samples were left unwashed after staining. Other researchers have reported the use of antigen negative EVs as negative controls for setting background fluorescence gates (58). However, because background fluorescence can often vary from individual to individual, it may be inappropriate to apply the gates created from a single sample of entirely different origin to all clinical samples in the study. Furthermore, this method requires the use of twice the amount of antibodies as well as a working bank of EVs known to be negative for all antibodies in question, which poses logistical difficulties.

The use of a lysis method is an established practice in EV analysis for identifying false positive events (55); however its utility as a replacement for isotypes in determining background fluorescence has not yet been described. In our research, we found that the lysis technique for gate placement worked as well or better than isotypes across all antibodies tested. It should be noted, however, that while using lysed samples for predicting background worked for our purposes, it may not necessarily be the best solution in all situations. One limitation of the lysis method is its inability to identify nonspecific binding of antibodies to EVs or other lipid vesicles such as chylomicrons. Furthermore, because lysis is not EV specific, some non EVs may become lysed and some small EVs may be resistant to lysis. For some, using a combination of the lysed sample and its isotype might work better than either alone for providing the best indication of background fluorescence for gate placement.

Washing After EV Staining

Removing unbound antibody after staining EVs often requires the use of lengthy, multi step washing procedures such as ultracentrifugation or sucrose fractionation. In an attempt to develop a protocol suited to high throughput sample analysis, we developed a technique using filters to wash EVs post staining. After staining with pre filtered antibody panels, EVs were originally added atop 0.2 μm centrifugal filters with 300 µL PBS and centrifuged at low speed ($\sim$600*g*) for 10 30 sec. After testing a variety of PPP samples it was found that some required centrifugation at 800*g* for 2 5 min, which was the final protocol we adopted. The tops of the filters were resuspended in 400 µL PBS and analyzed using FCM. Figure 4 shows the results using antibodies for which resolution was improved after implementing post stain filtration to remove unbound antibody. Similar results were obtained with each antibody tested (data not shown). Assay reproducibility was tested across seven different markers in three experiments performed in triplicate on PPP from a single normal donor. Coefficients of variation (CVs) for non post stain filtered samples were 38.7, 15.6, 11.6, 28.9, 40.1, 12.8, and 2.4 for the markers CD14, CD16, CD19, CD152, CD235a, CD108, and CD41a, respectively. CVs for post stain filtered samples were 10.9, 40.7, 4.9, 11.3, 19.6, 16.4, and 7.5 for the same markers, which was not significantly different from the samples that were not post stain filtered (P 0.45).

Of the washing techniques currently used to remove unbound antibody post staining, all are time consuming, multi step procedures not suitable for high throughput analysis. The most common methods, ultracentrifugation and sucrose fractionation, require long processing times and expensive equipment. Density gradient techniques described in the literature require 14 to 20 h centrifugations at forces of up to 192,000*g* (3,70). Here, we report the use of a novel filtration technique for reducing background fluorescence that is simple, fast, and effective. It should be noted that a significant limitation of this method is the loss of particles such as exosomes and small EVs that are small enough to pass through the 220 nm filter. This is an important consideration, as increasing evidence suggests that these very small EVs comprise the active/functional fraction of EVs as a whole, at least in some settings (68). One solution to this limitation would be to recover the filtrate, couple the smaller EVs to beads to allow washing, and analyze bead bound small EVs; however, this would limit one's ability to measure co expression of multiple antigens on single EVs. While the increase in signal to noise ratio is of obvious benefit, the loss of smaller

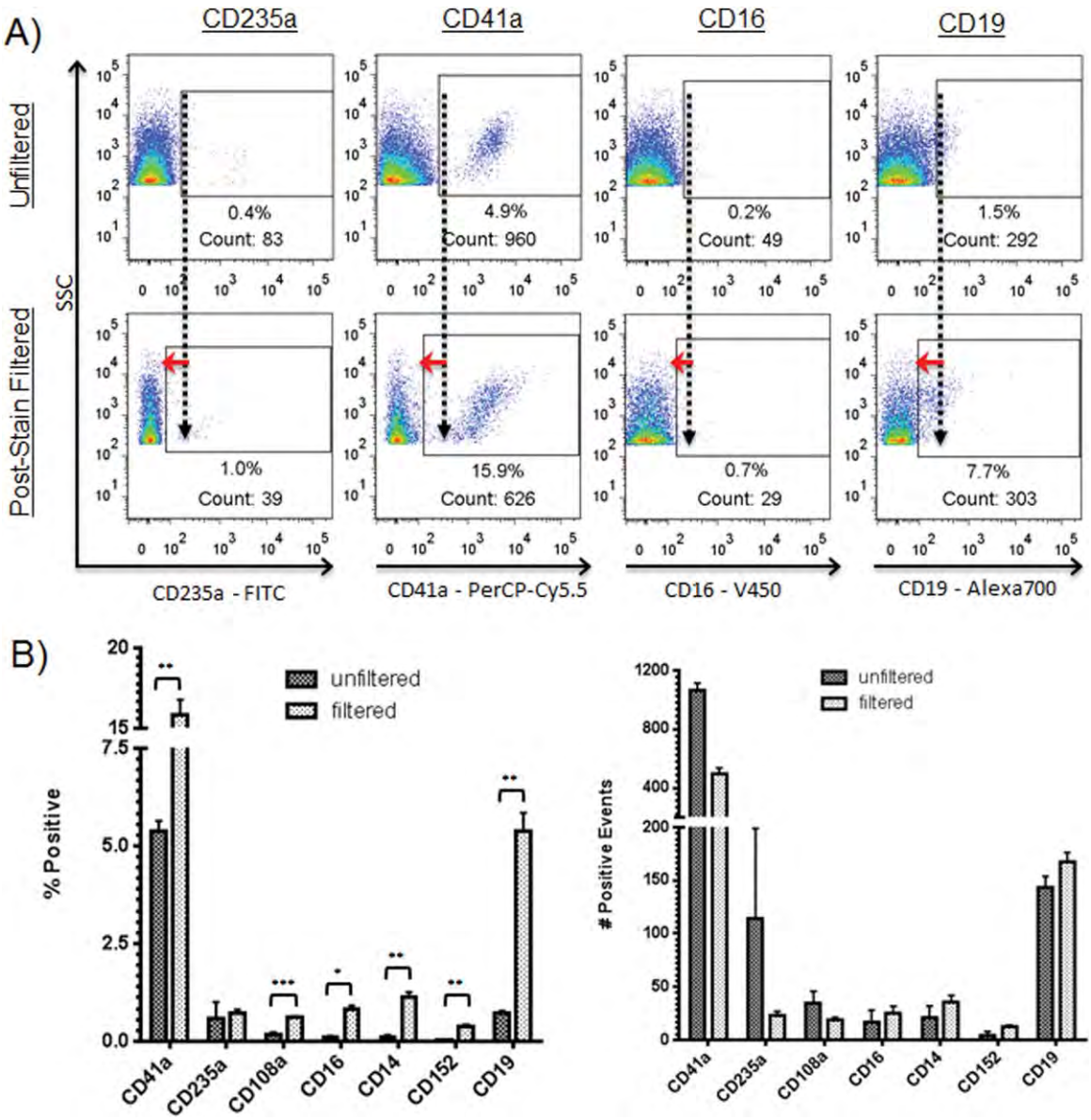

Figure 4. Effect of post stain filtration. EV samples were acquired before and after post stain filtration of aliquots of the same sample. Briefly, stained EV samples were added atop a 0.22 μm filter, centrifuged, and the EVs remaining on top resuspended in 400 μL PBS and read using FCM. **A**: Representative flow cytometric dot plots showing effect of post stain filtration. Events shown are within the FSC/SSC EV gate. Values show percentages of positive events. EV samples were read before and after post stain filtration. Antibodies were added to EVs for 30 min then either diluted and read immediately using FCM (top row) or post stain filtered and then read (bottom row). Red arrows highlight the difference in background staining intensity. **B**: Effect of post stain filtration on seven CD markers using EVs from normal donors run in triplicate in three experiments. $***P < 0.0005$, $**P < 0.005$., $*P < 0.05$. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

EVs represents a significant limitation when considering any washing method (63).

Effect of EV Concentration on Assay Sensitivity

It has been described that the flow cytometer can detect multiple small vesicles simultaneously illuminated by the cytometer's laser beam, counting them as a single event (50). The phenomenon of coincidence detection would presumably be more pronounced in samples with higher concentrations of EVs, which could affect the number and type of events detected. Figure 5 shows the results of six different dilutions of PPP on EV detection using FCM. PPP from five healthy donors was stained for CD41a, then post stain filtered and resuspended in PBS. The samples were then serially diluted, and each was read for 60 sec on a flow cytometer. While the percentage of positive events was fairly unpredictable at very low EV concentrations, the number of positive events detected within a fixed time frame decreased proportionally with dilution factor, yielding approximately the same calculated number of CD41a+ events at each dilution. CVs of CD41a+

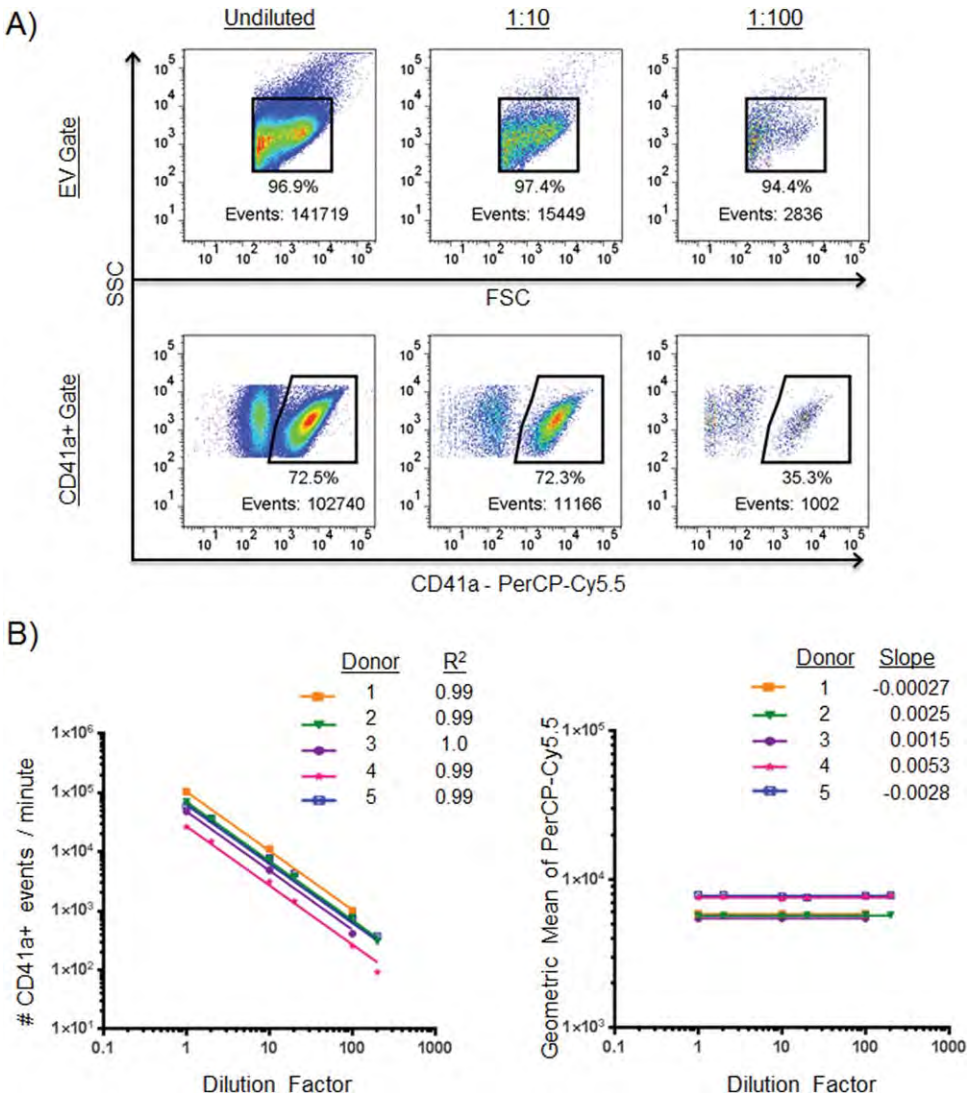

Figure 5. Effect of EV concentration on CD41a marker detection. **A**: Representative plots showing an undiluted sample (left column) and the same sample diluted 1:10 (middle column) and 1:100 (right column). Events shown in the bottom row are within the FSC/SSC EV gate above. Events numbers within positive cell marker gates are more reliable than percentages within those gates or event numbers within total EV gates. **B**: Effect of EV concentration on geometric mean and number of CD41a positive events detected by the cytometer. Samples of PPP from five healthy normal donors were concentrated using filters then serially diluted to six different concentrations. The left plot shows the relationship between EV concentration and number of events. *R* squared values indicate goodness of fit of each donor as determined by nonlinear regression analysis with slope constrained to −1.0. The right plot shows the relationship between EV concentra tion and the PerCP Cy5.5 geometric mean intensities of CD41a+ events at each of the six dilutions. Slopes were determined by nonlinear regression analysis.

calculated counts were 5.4, 9.8, 9.6, 16.3, and 8.4 for the five donors across all the dilutions, comparable to the CV of 7.5 for replicates of one sample tested undiluted presented in Fig ure 4. These data show that across the concentration range of ~100 100,000 positive events collected, the calculated count of EVs positive for CD41a did not vary substantially, implying that co incident particle detection did not play a significant role in detecting particles >200 nm at the concentrations tested.

Because coincidence detection is dependent on the num ber of surrounding EVs in a given sample, we thought it might be necessary to normalize the EV concentration of each sam ple prior to analyzing with FCM. However, we found no evi dence to support coincident particle counting in the dilution range we tested. In our dilution experiments we found that the percentages of positive events were equal and event counts were proportional to dilution factor between 1×10^5 and 1×10^6 EV/µL. With each 10 fold dilution, the number of positive events detected predictably dropped by a factor of 10. At higher dilutions, however, the percentages of events staining positive for a given marker became much less reliable due to a constant number of artifactual events in the denominator,

while event counts staining positive for a given marker remained consistent with dilution factor. Whereas percentage varied considerably at higher dilutions (due to the noise mak ing up a larger proportion of the denominator at higher dilu tions), the number of positive events was consistent and reliable. With the analysis of these dilution experiments, we gained a new understanding of our results and learned that the best parameter to record was the number of positive events in a set time period, not percentage of positive EV events. With this finding, we changed our recording methods to place new emphasis on number of positive events collected within a fixed time frame, rather than percentage of the total EV population collected being positive for the marker in ques tion. Because of the influence of background noise at low EV concentrations, we concluded that counting the number of positive events within a fixed time frame yielded more repro ducible results than using the percentage of the total EV popu lation being positive for the marker in question. The CVs in these dilution experiments (between 5.4 and 16.3, mean 9.9) were similar to the CV we observed in our reproduci bility experiments (7.5), thus concentration is unlikely to play a huge role in our sample to sample variability.

Conclusion

Here, we have presented optimization techniques that are especially well suited for analyzing EVs from a high volume of clinical samples. In particular, we showed that 1) filters are a good alternative to centrifugation for removing antibody aggregates before staining, 2) lysed samples are a useful alter native to isotypes for setting gates to exclude background fluorescence, 3) filters can be used to "wash" samples post staining thus providing a faster alternative to ultracentrifuga tion and sucrose gradient fractionation, and 4) normalization of EV concentration prior to staining is unnecessary in the concentration range we examined. Methods for EV analysis, while considerably improved over the last decade, are still a work in progress. Ultimately, the best methods for analyzing EVs will depend on the individual lab's needs and tools avail able to the researcher. The techniques described here will assist with eliminating the antibody aggregates commonly found in commercial preparations, increasing signal to noise ratio, and setting gates in a rational fashion that minimizes detection of background fluorescence.

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Author Contributions

H.C.I. designed and performed the experiments, analyzed and interpreted the data, and wrote the manuscript. A.D. designed and performed the experiments. A.S. and J.L. coordi nated collection of study participant samples. P.C.S. coordi nated collection of study participant samples and edited the article. P.J.N. designed the experiments, interpreted the data, and edited the article.

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Video Article

Techniques for the Analysis of Extracellular Vesicles Using Flow Cytometry

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Abstract

Extracellular Vesicles (EVs) are small, membrane-derived vesicles found in bodily fluids that are highly involved in cell-cell communication and help regulate a diverse range of biological processes. Analysis of EVs using flow cytometry (FCM) has been notoriously difficult due to their small size and lack of discrete populations positive for markers of interest. Methods for EV analysis, while considerably improved over the last decade, are still a work in progress. Unfortunately, there is no one-size-fits-all protocol, and several aspects must be considered when determining the most appropriate method to use. Presented here are several different techniques for processing EVs and two protocols for analyzing EVs using either individual detection or a bead-based approach. The methods described here will assist with eliminating the antibody aggregates commonly found in commercial preparations, increasing signal–to-noise ratio, and setting gates in a rational fashion that minimizes detection of background fluorescence. The first protocol uses an individual detection method that is especially well suited for analyzing a high volume of clinical samples, while the second protocol uses a bead-based approach to capture and detect smaller EVs and exosomes.

Video Link

The video component of this article can be found at http://www.jove.com/video/52484/

Introduction

Extracellular Vesicles (EVs) are small, membrane-derived vesicles found in bodily fluids that are highly involved in cell-cell communication and help regulate a diverse range of biological processes. Analysis of EVs using flow cytometry (FCM) has been notoriously difficult due to their small size and lack of discrete populations positive for markers of interest. Methods for EV analysis, while considerably improved over the last decade, are still a work in progress. Unfortunately, there is no one-size-fits-all protocol, and several aspects must be considered when determining the most appropriate method to use. Presented here are several different techniques for processing EVs and two protocols for analyzing EVs using either individual detection or a bead-based approach. The methods described here will assist with eliminating the antibody aggregates commonly found in commercial preparations, increasing signal–to-noise ratio, and setting gates in a rational fashion that minimizes detection of background fluorescence. The first protocol uses an individual detection method that is especially well suited for analyzing a high volume of clinical samples, while the second protocol uses a bead-based approach to capture and detect smaller EVs and exosomes.

EVs, also known as microparticles, are small, membrane-derived vesicles found in bodily fluids that are involved in cell-cell communication and help regulate a diverse range of biological processes[1]. Through expression of various surface markers and/or direct transfer of biological material, EVs are able to alter the function of recipient cells to play either activating or suppressing roles in intercellular communication[2–4]. Clinically, platelet-derived EVs are known to have strong anticoagulant activity[5], while others have been shown to contribute to a wide range of conditions, from promoting tumor metastasis[6] to protecting against disease[7]. EVs can be classified into smaller categories of cell-derived vesicles such as exosomes and microvesicles (MVs), depending on their size and mechanism of generation[8]. The nomenclature of cell-derived vesicle subpopulations continues to be a topic of ongoing debate[8,9], however, exosomes are generally described as small, 40 to 100 nm particles derived from endosomal fusion with the plasma membrane, while MVs are larger 100 to 1,000 nm particles formed by shedding of the plasma membrane[10]. Here, the general term “EVs” will be used to refer to all types of extracellular biological vesicles released by cells.

Isolation of EVs from whole blood is a multi-step procedure and many different processing variables have been shown to affect EV content, including storage temperature and duration[11,12], anticoagulant/preservative used[13] and centrifugation method used[14]. A need for standardization of these variables has led to recommendations by the International Society on Thrombosis and Haemostasis Scientific and Standardization Committee (ISTH SSC) for proper blood processing and EV isolation procedures[15,16], yet there exists no consensus among researchers on the optimal protocol to use [12]. Most agree, however, that tightly controlled pre-analytical variables are crucial for accurate and reproducible data.

In order to analyze EVs, researchers have utilized various methods, including transmission electron microscopy[17], scanning electron microscopy[18,19], atomic force microscopy, dynamic light scattering[20,21] and western blotting[22,23]. While FCM is the method of choice for many researchers[9,24–26] due to its high throughput capabilities, analysis of EVs using FCM has been notoriously difficult due to their size and lack of discrete positive populations[27–32]. Compared to analysis of cells, the small size of the EVs results in 1) less fluorescence emitted due to the

fewer number of antigens per particle and 2) limited feas bility of post-stain washing, which is necessary to reduce background fluorescence. Common challenges among researchers include signals arising from immunoglobulin aggregates[27,28] and self-aggregation of antibodies[29]. Furthermore, the long processing times and lengthy washing/isolation procedures used by many of the current protocols[33,34] require multi-day time commitments to analyze a small number of samples, making them less than ideal for high throughput applications. Some researchers forgo a wash step altogether, rendering traditionally used FCM negative controls such as fluorescence minus one (FMO) and ant body isotypes useless for accurately assessing background fluorescence[30].

Our protocols address three common problems that can impede proper FCM analysis of EVs: signals arising from ant body aggregates and other non-vesicles, difficulty in removing unbound antibody, and lack of discernible positive populations. The techniques described here will assist with eliminating the antibody aggregates commonly found in commercial preparations, increasing signal–to-noise ratio, and setting gates in a rational fashion that minimizes detection of background fluorescence. Two different detection methods are presented here: the first protocol uses an individual detection method that is especially well suited for analyzing a high volume of clinical samples, while the second protocol uses a beads-based approach to capture and detect smaller EVs and exosomes.

Protocol

NOTE: The following protocols have been performed in compliance with all institutional, national and international guidelines for human welfare. All human subject samples were tested under an institutional review board (IRB)-approved protocol and with informed consent of the subjects.

1. METHOD A: Individual Detection Method

1.1) Processing of Blood Sample/Isolation of EVs

1. Draw blood from donor/patient into two 10 ml glass tubes containing 1.5 ml of ACD-Solution A or other suitable anticoagulant and process immediately (within 30 min max) using the following 2-step differential centrifugation protocol.
   NOTE: This protocol will yield approximately 10 ml of platelet poor plasma (PPP) from the combined ~17 ml of blood drawn. If more or less PPP is needed, the number of tubes of blood collected may be adjusted accordingly.
2. Centrifuge the samples at 1,500 x g for 10 min at RT to separate the plasma from the buffy coat and red cells. Transfer 1.2 ml aliquots of the plasma supernatant to 1.5 ml centrifuge tubes, being careful not to disturb the bottom layers containing the buffy coat and red cells.
3. Spin at 13,000 x g for 10 min at RT to remove platelets and large cell fragments. Carefully transfer the PPP, leaving behind 200 µl to avoid disturbing the pellet and add the PPP to a new tube.
4. At this point, use PPP immediately for analysis or transfer in 1.0 ml aliquots to new 1.5 ml centrifuge tubes and store at -80 °C for up to two years for later analysis (refer to **Figure 1A** for overview).
5. If purified EVs are needed for functional experiments, transfer 6 ml of the PPP to an ultracentrifuge tube and add 28 ml of 0.2 µm-filtered phosphate buffered saline (PBS). Spin for 60 min at 100,000 x g at RT using an ultracentrifuge equipped with a swinging bucket rotor. Aspirate supernatant and resuspend EV pellet in 1.5 ml media.
   NOTE: For highest reproduc bility, blood samples should be processed as consistently as possible from donor to donor. Any variation in EV isolation method could significantly impact the number and type of EVs detected.

1.2) Preparing Samples for Analysis

NOTE: From this point on, the steps explain a high throughput protocol for analyzing 12 samples for 14 markers in 3 panels. However, other combinations of antibodies can be used here; the protocol can be adapted to study other EV populations by substituting the suggested markers for those of interest.

1. Remove 12 samples from freezer (if stored at -80 °C) and thaw at 37 °C.
2. Pipette contents up and down several times to mix. Remove 320 µl from each sample and add to the top row of a 96 well plate.
   NOTE: A width-adjustable multi-well pipet is extremely helpful for this and many other steps throughout the assay, particularly when analyzing multiple samples at once.

1.3) Staining EV Samples

1. Prior to staining, filter all antibodies (Abs) to remove aggregates, which can cause positive signals.
   1. Combine ant bodies to be used in each of the 3 panels into separate 0.22 µm centrifugal filter tubes and centrifuge using a fixed angle single speed centrifuge (~750 x g) at RT for 2 min, or until all of the Ab mixture has passed through the filter and no antibody liquid remains on the surface of the filter. Store Ab cocktails in the fridge for up to two weeks but re-filter each time before use.
2. Add the appropriate amount of filtered Ab mixture to each well in row 2 (*e.g.*, samples in Panel I are stained with 2 µl of each Ab, so a total of 12 µl of the filtered Ab cocktail is added per well to row 2). Refer to **Figure 1B** for an outline of the suggested plate map. Repeat these additions to the rows beneath if more panels are run (here, add 8 µl/well of the Panel II cocktail to row 3 and 11 µl/well of the Panel III cocktail to row 4; refer to Materials List for specific panel information).
3. Using the multichannel pipet, mix the PPP samples in row 1 up and down and transfer 100 µl from the wells in row 1 to the wells in row 2. Mix up and down. Change tips and repeat, transferring 100 µl from row 1 to rows 3 and 4. Incubate at 4 °C for 30 min.

1.4) Washing MV Samples

1. Remove the 96-well plate from 4 °C and transfer to biological safety cabinet. Using a multichannel pipette, add 220 µl of PBS/well to rows 6-8 (to be used for rinsing/washing the wells containing stained PPP).

2. Transfer the contents of each well to pre-labeled centrifugal filter tubes using the width-adjustable multichannel pipet (For 12 samples, with 3 panels of antibodies, 12 x 3 = 36 filter tubes will be needed). Using the same tips, remove 200 µl of PBS from the wash rows and add to the corresponding wells from which PPP was just removed.
3. Mix up and down to rinse the wells and transfer the rinse solution to the same filters to which the PPP was previously added. Close tops of centrifugal filters. Change tips.
4. Repeat this process with the remaining stained samples until all stained PPP samples have been transferred along with their rinse solutions to centrifugal filters.
5. Transfer the centrifugal filters to a fixed rotor centrifuge and spin at 850 x g for 3 min at RT.
   NOTE Ensur tha n liqui remain o th filte tops Afte centrifugation th filte shoul appea t b “dry wit n visibl flui layer remainin o top Whil unlikely certai PP sample ma requir longe centrifugatio tim t effectivel mov throug th filter.
6. Remov th centrifuga filte tube an retur t th biologica safet cabinet Usin th multichanne pipet resuspen th top o th filter in 30 µ o PBS Transfe th resuspende content t pre-labele tube fo immediat FC analysis.
   NOT : t s ve y importa t o ke p t e for e a d numb r f pipet e plung r depressio s consiste t amo g sampl s o avo d sample-to-samp e variatio . Th s shou d ideal y e do e usi g n electron c pip t th t h s be n programm d o pip t p a d do n a specif c volume (*e.g* , 2 0 µ) n exa t numb r f tim s (*e.g* , 8 time) f r ea h sample.

1.) Cytomet r Setup

1. Op n t e F M softwar . Pri r o experime t setu , perfo m dai y instrume t calibrati n a d set p usi g instrume t set p bea s (following manufacturer s instructions).
2. f V sampl s ha e be n stain d wi h mo e th n o e a tibo y a d multip e fluorochrom s a e o e measur d t onc , calculate compensati n valu s s follows:
   1. A d 2 dro s f compensati n bea s o pre-label d tub s 1 tu e f r ea h fluorochrome-conjugat d antibod) a d a d the recommend d amou t f a tibod . A d 2 dro s f negati e compensati n bea s o anoth r tu e o u e s t e unstained compensati n control.
   2. Incuba e t 4 C f r 0 mi , wa h wi h P S a d resuspe d n 4 0 l f PBS.
   3. Usi g t e fl w cytomet r softwa e includ d wi h t e instrumen , r n ea h compensati n tu e a d adju t fluoresce t voltag s o place ea h pe k t approximate y 1 ^{4} n a 5-deca e l g scal . Ensu e th t t e fluorescen e pe k s highe t (brightes) n i s o n fluorescent chann l compar d o a l oth r channel , a d adju t voltag s f fluoresce t paramete s aga n f necessar . R n ea h individually-stain d co p tu e a d captu e t lea t 5,0 0 even s p r tube.
   4. Sele t t e t b “Experiment ” th n sele t “Compensation ” th n sele t “Calcula e Compensatio ” o app y compensati n valu s o all samples.
3. S t t e forwa d scatt r (FS) a d si e scatt r (SS) volta e paramete s o l g sca e a d sele t t e lowe t threshol s allow d y the cytomet r (FSC=2 0 a d SSC=20) f r each.
4. Whi e runni g a tu e f 0. 2 µm-filter d PB , adju t t e F C & S C voltag s o t e highe t valu s th t exclu e t e majori y f background noi e (*i.e* , ju t bel w t e volta e thresho d t whi h eve t ra e surpass s 5 events/sec).
5. Nex , r n a tu e containi g 0 2 m – 1 0 m bead , dilut d n P S f necessar . n n F S v . S C plo , dr w a ga e arou d t e bead populati n o captu e even s betwe n 0 2 m a d 1 0 µ . Alternativel , n n SSC H histogra , dr w a ga e o inclu e a l even s small r than t e 1 0 m beads.
6. S t t e cytometer s fl w ra e o “L ” (approximate y 8- 2 µl/min . Usi g t e bea s tu e (r oth r tu e containi g a kno n concentrati n of bead) adju t t e fl w ra e di l n t e cytomet r unt l t e eve t ra e reach s approximate y 2 0 events/se . Re d a l samp e tub s t the sa e fl w ra e a d u e t e sa e be d concentrati n n futu e experimen s o ensu e th t fl w rat s rema n consiste t betwe n runs.
7. R n a tu e f rainb w fluoresce t particl s dilut d n PB . Acqui e 5,0 0 event . Reco d t e me n intensi y valu s f r FS , SS , a d each col r channe . U e the e valu s o adju t voltag s n futu e experimen s o ensu e th t fluorescen e intensiti s rema n consiste t between experiments.

1.) Samp e Reading

1. S t t e cytometer s fl w ra e o “L ” (approximate y 8- 2 µl/mi) a d r n ea h samp e f r exact y 1 r 2 min.
2. Aft r t e fir t readin , a d 0 l f 1 % NP- 0 o ea h sampl , pipet e p a d dow , a d re-re d f r t e sa e amou t f ti e (eith r 1 r 2 mi) o all w f r t e subtracti n f positi e even s detect d n t e lys d samp e ov r n equ l ti e frame.
   NO E: It is extrem ly import nt t at samp es be mi ed us n a pi et rat er t a a vort x. In ur experien e, vortex ng an ca se self-aggregat on of s me antibodi s, lead ng to EV-mimick ng posit ve events.
3. O ce ll samp es h ve b en re d, exp rt ll . cs fi es i t a separ te f le to be u ed or furt er analy is us ng CM analy is software.

1 7) D ta Analysis

1. O en he CM analy is softwa e. Imp rt ll of he . cs fi es i t a ew experim nt file.
2. O en he beads-o ly tu e. In he FS -A s. SS -A pl t, d a a g te aro nd ll be ds si ed betw en .2 µm nd .0 m. T is is he EV ga e. D ag to dd to ll samples.
3. Us ng he ly ed samp es as negat ve contro s, d aw ga es at he e ge of backgro nd fluoresce ce in e ch fluoresce ce chan el us d. Drag fluoresc nt ga es i to he EV g te of e ch correspond ng non-ly ed sample.
   N TE At his p in i is lso us fu to exa ine ual fluores ent bi-param ter pl ts. Ro ket sh pe in the double-posi ive quad ant (see **Fi ure 8**), particul rl if the mar ers are k ow to re id on unrel ted ell ty es, ma be indica iv of arti act rom aggrega io or other vesicle-mimic ing events.
4 For ach fluores ent mar er, subt act the nu be of ev nt in the l sed sa ple rom the nu be of ev nt in the non-l sed sample. Optiona ly, di ide his nu be by the t tal nu be of EVs wi hin the non-l se EV at to g t % posi ive values.

2. METHOD B: Beads Method

2.1) Processing of Blood Sample/Isolation of EVs

1. Refer to the blood processing method described in Method A (Section 1.1).

2.2) Preparing Samples for Analysis

1. If desired, fractionate PPP or ultracentrifuged EVs into exosomes and microvesicles. Add 250 µl of PPP or ultracentrifuged EVs to 0.22 µm centrifugal filters and transfer to a fixed rotor centrifuge and spin at 750 x g for 2 min at RT.
   NOTE Ensur tha n liqui remain o th filte tops Afte centrifugation th filte shoul appea t b "dry wit n visibl flui layer remainin o top Whil unlikely certai sample ma requir slightl longe centrifugatio tim fo th flui t effectivel mov throug the filter.
2. Was uncoate µ polystyren bead (*e.g.* negativ Ab beads 2 wit RPM media an resuspen i ml Ad 6,00 bead to eac FAC tube T th negativ contro tube ad 40 µ o RPM medi alon t th beads T al othe tubes ad 20 µ o PP or ultracentrifuge EV (o thei fractions an 20 µ o RPM media.
3. Adjus th fina volum o al tube t 40 µ wit medi an incubat overnigh a ° o shaker.
4. Th nex morning was bead wit m o media Aspirat of th supernatant.
5. Bloc wit 5 bovin seru albumi (BSA i medi (40 µl fo h a ° o shaker.
6. Was bead wit m o media Aspirat an resuspen pelle i 10 µ o medium.

2.3 Stainin E Samples

1. Filte al antibodies Us th sam antibod panel use i Metho A o i desired creat differen combinatio o antibodie a lon as thei fluorochrome ar compatibl wit on another.
2. Combin al antibodie t b use i singl pane int 0.2 µ centrifuga filte tube Centrifug usin fixe angl singl speed centrifug fo mi o unti al o th A mixtur ha passe throug th filte an n antibod liqui remain o th surfac o th filter.
3. Ad appropriat volum o filtere antibod cocktai t al tube an incubat fo 3 mi a °C.
4. Was bead wit m o media resuspen i 40 µ o medi an ru immediatel (o withi th sam day o flo cytometer.

2.4 Cytomete Setu an Sampl Reading

1. Ope th FC software Prio t experimen setup perfor dail instrumen calibratio an setu usin instrumen setu bead (following manufacturer' instructions).
2. I E sample hav bee staine wit mor tha on an ibod an multipl fluorochrome ar t b measure a once calculate compensatio value a follows:
   1. Ad drop o compensatio bead t pre-labele tube (tub fo eac fluorochrome-conjugate antibody an ad the recommende amoun o an ibody Ad drop o negativ compensatio bead t anothe tub t us a th unstained compensatio control Incubat a ° fo 3 min was wit PB an resuspen i 40 µ o PBS.
   2. Usin th FC software ru eac compensatio tub an adjus fluorescen voltage t plac eac pea a approximatel 10 on 5-decad lo scale Ensur tha th fluorescenc pea i highes (brightest i it ow fluorescen channe compare t al other channels an adjus voltage o fluorescen parameter agai i necessary Ru eac individually-staine com tub an captur at leas 5,00 event pe tube.
   3. Selec th ta "Experiment, the "Compensation, the "Calculat Compensation t appl compensatio value t al samples.
3. Chang th FS an SS voltag parameter t lo scal an selec th lowes threshold allowe b th cytomete (FS 20 an SS = 200 fo each.
4. Ru samples gat o th single bead populatio an acquir 2,00 event i thi gate Expor .fc files.
5. Us FC analysi softwar t analyz .fc files Gat o single beads Calculat th geometri mea fluorescen intensit (MFI fo each fluorochrom an compar wit th MF o th negativ control.

Representativ Results

Figur outline th overal processin schem fo th isolatio an detectio o EV usin eithe th bead-base metho o individual detectio method Individua detectio o EV usin FC work wel fo analyzin large EV bu mos cytometer ar no capabl o individually detectin particle a smal a exosomes bead-base approac allow smal EV t b detected however ther ar drawback associated wit usin thi method a outline i **Tabl 1** Generally isolatio o EV usin ultracentrifugatio (wit o withou th additio o sucrose gradien fractionatio procedure i recommende whe EV ar neede fo functiona assays Ultracentrifugatio remove impuritie including seru protein an othe solubl contaminant fro th plasma whic ca affec functiona experimenta outcomes However ultracentrifugation i tim consumin an ma alte E quantit an quality[12].

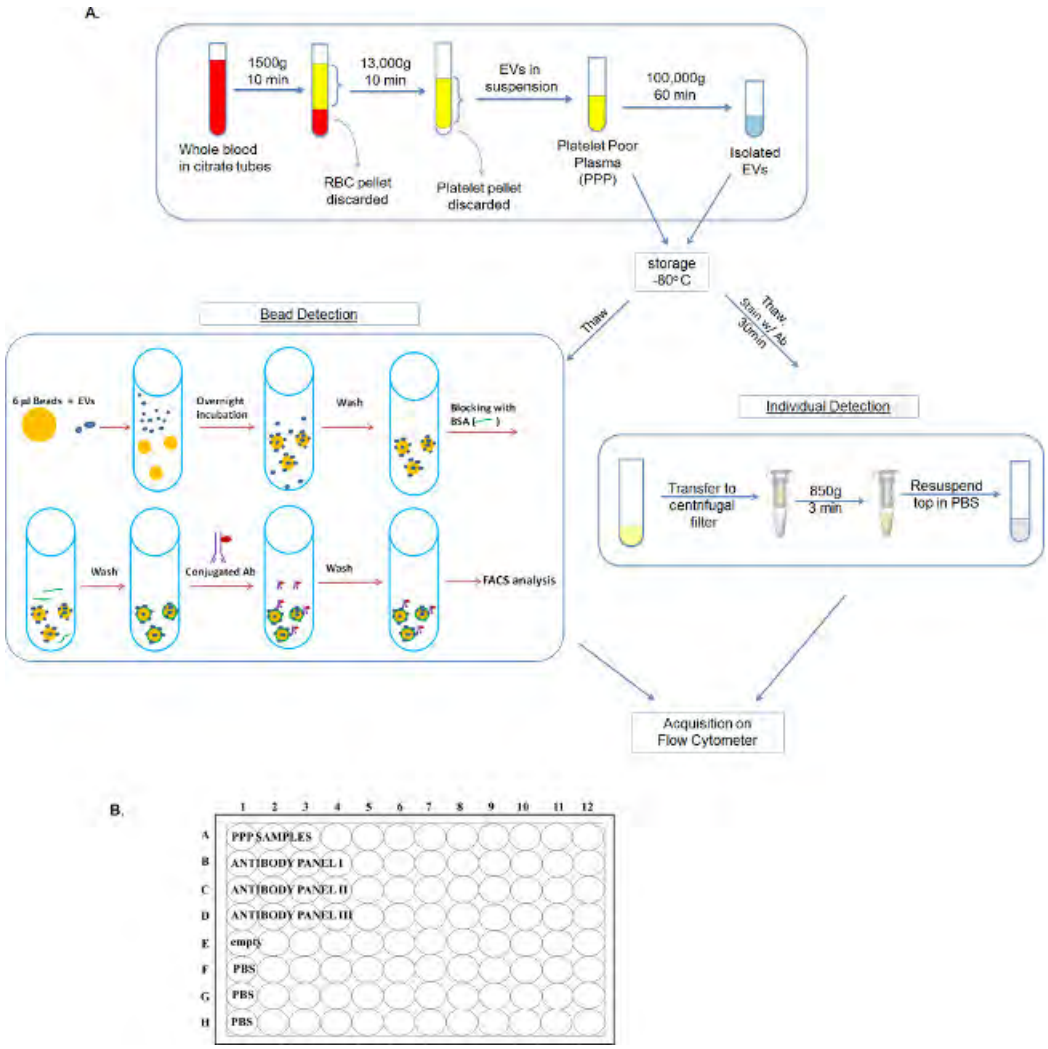
Expecte result fo th tw detectio assay ar depicte i **Figure 2-3** Fo th individua detectio assay th lyse contro (botto row, **Figur 2** i use t se gate fo th correspondin non-lyse sampl (to row **Figur 2**) Th majorit o event shoul fal withi th E gate. Quadran gate shoul no revea doubl positiv event whe th tw marker i compariso ar no normall foun o th sam cell Th right biparamete plot i **Figur** sho th marker CD108 an CD235a whic ar tw re bloo cel marker know t coexis o cells Here, o EVs ove hal o th positiv event ar positiv fo bot markers a expected I th sam way cel surfac marker know t resid on th sam cel shoul sho simila pattern o doubl positivit o EVs Th cente biparamete plot sho E expressio o tw marker that ar know *no* t coexis o cells I thi analysi o CD235 (re bloo cel marker an CD41 (platele marker) th EV sho distinct, separate positiv populations whic i expecte sinc the com fro differen cel types Whe lysed positiv event shoul disappear. I general an positiv event remainin afte lysi indicat th presenc o signa comin fro non-vesicl particles aggregates and/or

detergent-resistant EVs. **Figure 3** shows expected results using the bead-based detection method. Unlike the individual detection method, these data cannot/should not be viewed in bi-parameter plots. In the upper dot plots, no separation between the positive and negative populations exist, and events appear in the double positive quadrants even though they aren't normally found on the same cell types due to the fact that both types of EVs will bind to a single bead. For the bead-based detection method, data are best analyzed using histogram overlays with the negative control (depicted underneath the dot plots). Positivity is measured using a marker's MFI (mean fluorescence intensity) and compared directly with that of the negative control. If a sample is positive for the marker in question, its MFI will be higher than the negative control. The negative control for the bead method is simply beads blocked with BSA (no EVs added), which have been stained with the same antibodies and washed alongside the EV-coated beads. A comparison of expected results using the two methods can be seen in **Figure 4**.

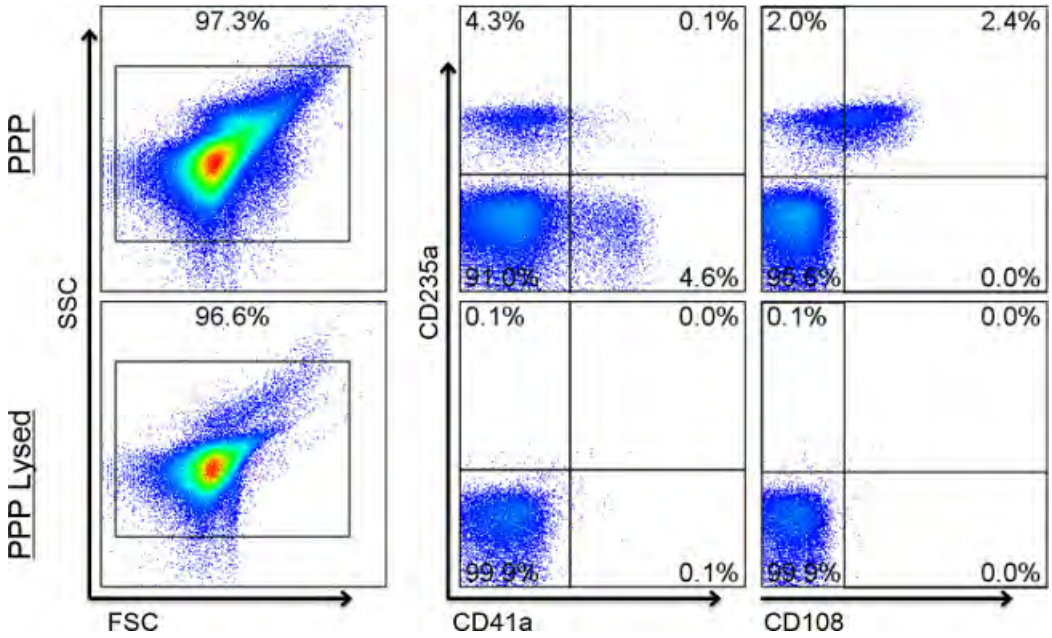
The ability of the individual detection assay to properly assess EV phenotypes relies heavily on correct gating to separate Ab-positive events from background fluorescence. Therefore, it is critical to choose a negative control that most appropriately mimics/predicts background fluorescence for a given sample. When stained EVs are not washed before reading, commonly used negative controls (*e.g.*, isotypes) fail to accurately predict background fluorescence for all markers (**Figure 5A**). In these cases, if washing is not an option, lysed samples tend to work better for predicting background fluorescence. However, when stained EVs are washed before reading (using centrifugal filtration, in this case), both negative controls (isotypes and lysed samples) work well for predicting background fluorescence of a sample (**Figure 5A**). It should be noted, however, that while all negative controls "work," lysed controls are preferred because they provide additional information about a sample (*e.g.*, the presence of detergent-resistant, non-vesicle-related events and/or aggregates) that can result in non-EV positive signals and improperly inflate Ab counts. Furthermore, isotype controls can sometimes be unreliable, even in washed samples, as shown in **Figure 5B**, where the stained sample has fewer positive events than the same sample stained with matched isotype control antibodies.

Without thorough removal of unbound antibody, FCM dot plots of some EV markers are nearly impossible to interpret, appearing as clouds of dimly fluorescent particles indistinguishable from their highly fluorescent backgrounds (**Figure 6**, top plot). Washing stained samples using centrifugal filters enhances the separation between background and positive marker signals (**Figure 6**, bottom plot); however, small EVs and exosomes may be lost through the pores of the filter.

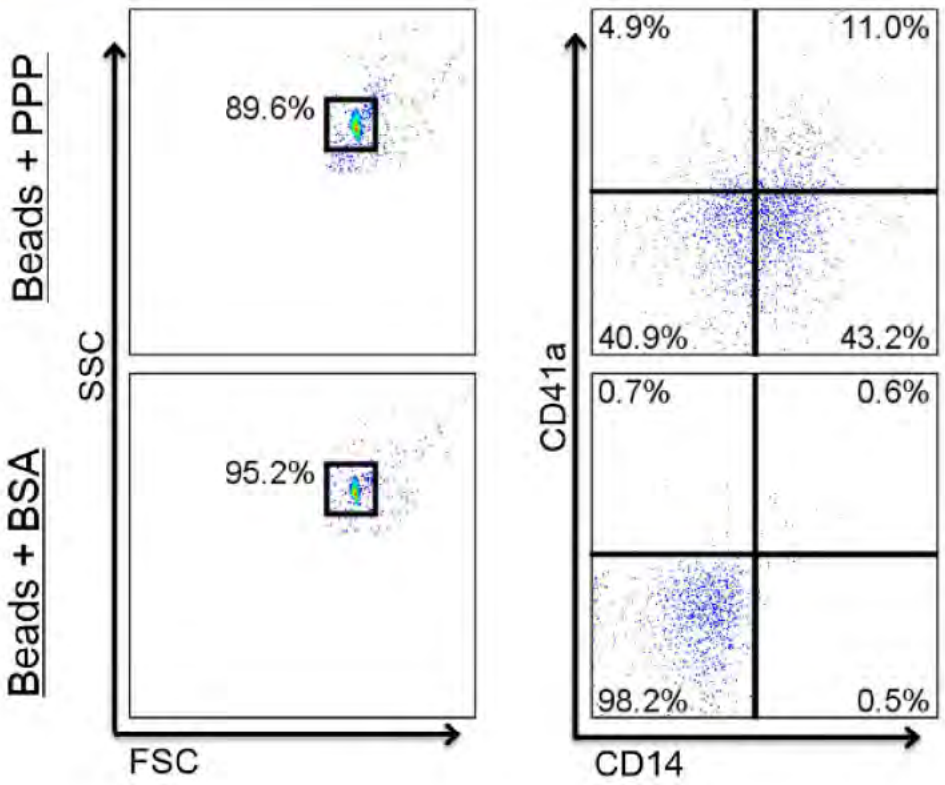
The use of a detergent lysis step reveals positive, vesicle-mimicking events from immune complexes and protein aggregates[21]. When PPP is analyzed using individual detection, encountering positive events that do not disappear with lysis is a fairly often occurrence. These detergent-resistant events often appear as suspicious, highly fluorescent diagonal signals in both single parameter and biparameter plots (**Figure 7**). Clinically, these protein complexes and/or insoluble immune complexes are more prevalent in patients afflicted with various diseases[21], such as rheumatoid arthritis[28], nephrotic syndrome[19], and systemic lupus erythematosus[29]. Therefore, depending on the objective of the research, one may wish to include or remove them from the analysis.Another way diagonal signals can form is by vortexing the samples, particularly after the addition of the lysis reagent (**Figure 8**). Samples should always be mixed up and down by pipet to prevent the formation of aggregates.



Figur 1 Overal processin schem fo th isolatio an detectio o EV usin eithe th bead-base metho o individua detection method (A Whol bloo i firs processe int PPP Fro there PP ca eithe b processe furthe usin ultracentrifugatio t yiel isolated EV o use as-i i th individua detectio o bead-base assays **(B** Outlin o suggeste plat ma fo hig throughpu sampl analysis usin th individua detectio method Pleas clic her t vie large versio o thi figure.



Figur 2 Expecte result fo Individua Detection Flo cytometr do plot sho representativ stainin o lyse an unlyse E samples. Value sho percentage o positiv events Th majorit o event fal withi th E gate Event show i th righ biparamete plot are withi th FSC/SS E gate o th left Th lyse sampl (botto row i use t se fluorescent-base gate fo eac correspondin (non-lysed sample Qua gate shoul no revea doubl positiv event whe th tw marker i compariso ar no normall foun o th same cell Here th biparamete CD235 an CD41 plo show distinc separatio betwee th EV expressin re bloo cel marker and thos expressin platele cel markers ikewise cel surfac marker know t resid o th sam cel shoul sho simila pattern o double positivit o EVs Th righ biparamete plo show tha ove hal o CD235a-positiv EV ar als positiv fo th secondar re bloo cell (RBC marker CD108a Whe lysed positiv event shoul disappear Positiv event remainin afte lysi indicat th presenc o signal comin fro non-vesicl o detergen resistan particle and/o aggregates.



Sample	MFI (geometric mean) CD41a	CD14
Beads + PPP	315.9	549.4
Beads + BSA (neg. ctrl)	67.1	53.3

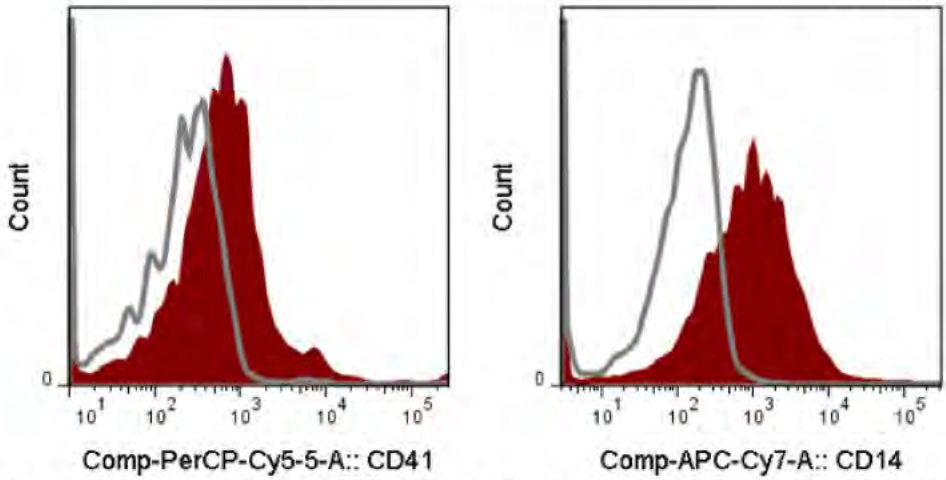
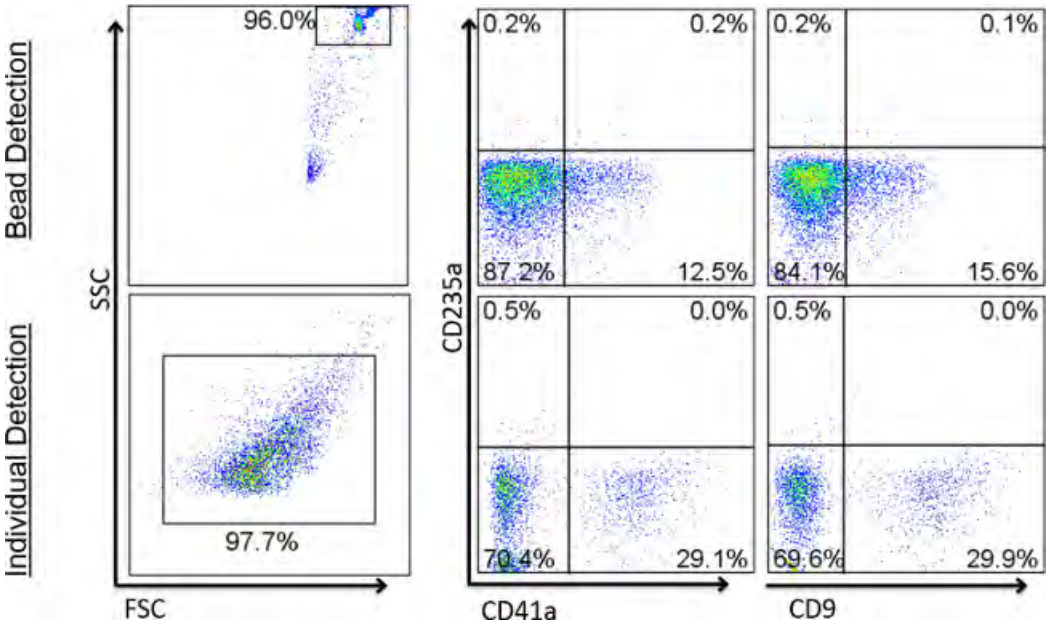
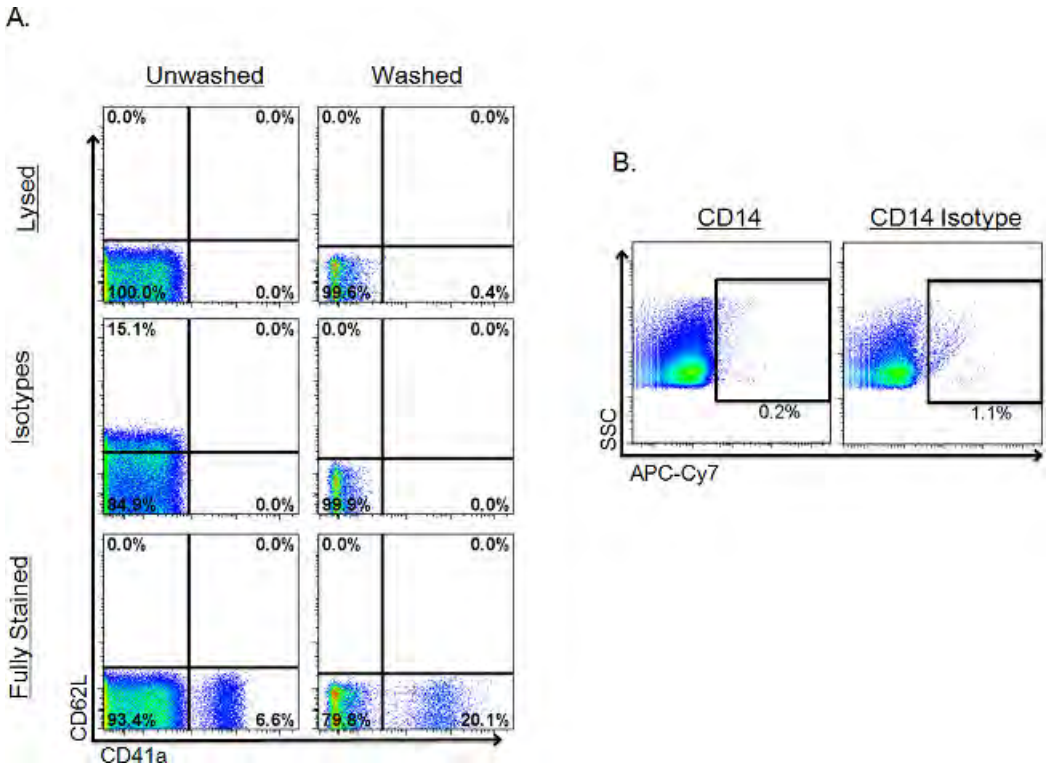


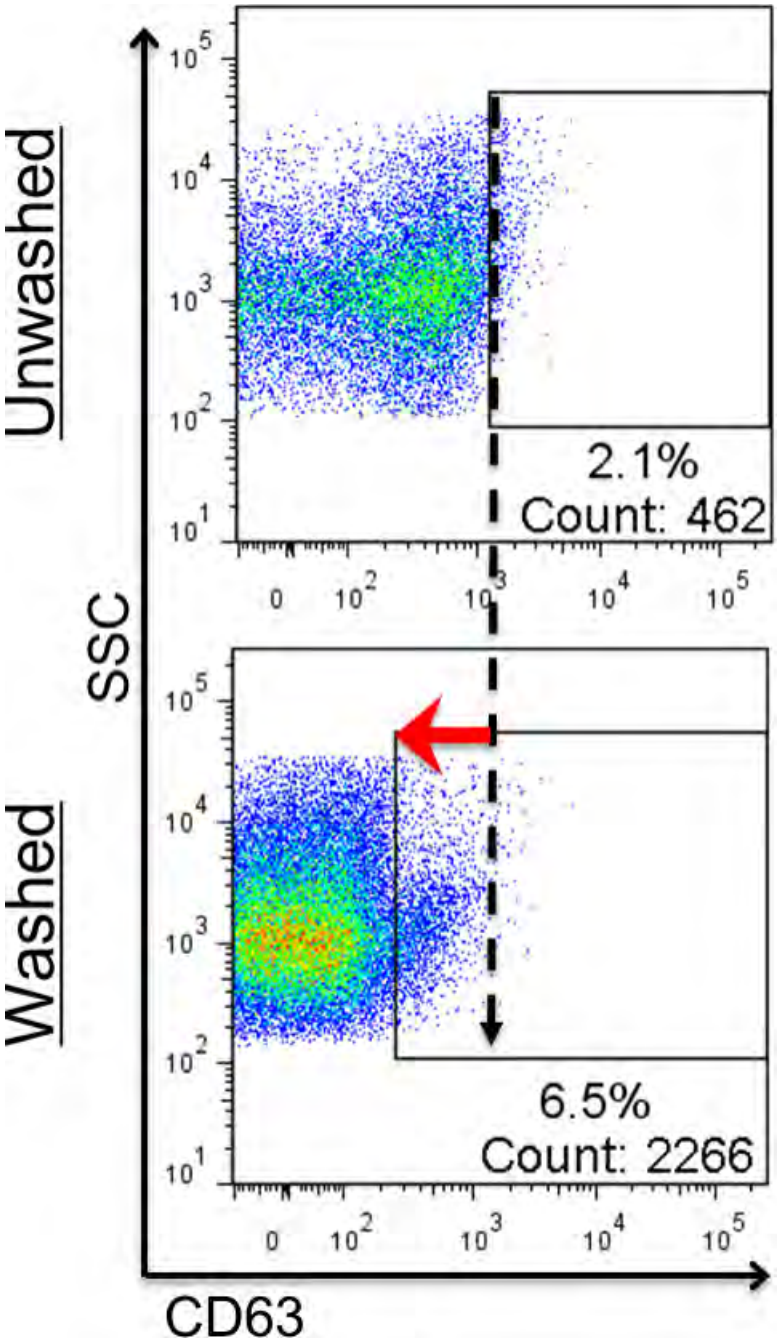
Figure 3: Expected results using the bead-based detection method. Flow cytometry dot plots show representative staining of EVs coupled to beads, as compared to beads blocked with BSA, which serves as the negative control. Values show percentages of positive events. Events shown in the right biparameter plots are within the FSC/SSC beads gates on the left. For the beads-based detection method, data are best analyzed using histogram overlays with the negative control (depicted underneath the dot plots). Positivity is measured using a marker's MFI (mean fluorescence intensity) and compared directly with that of the negative control.



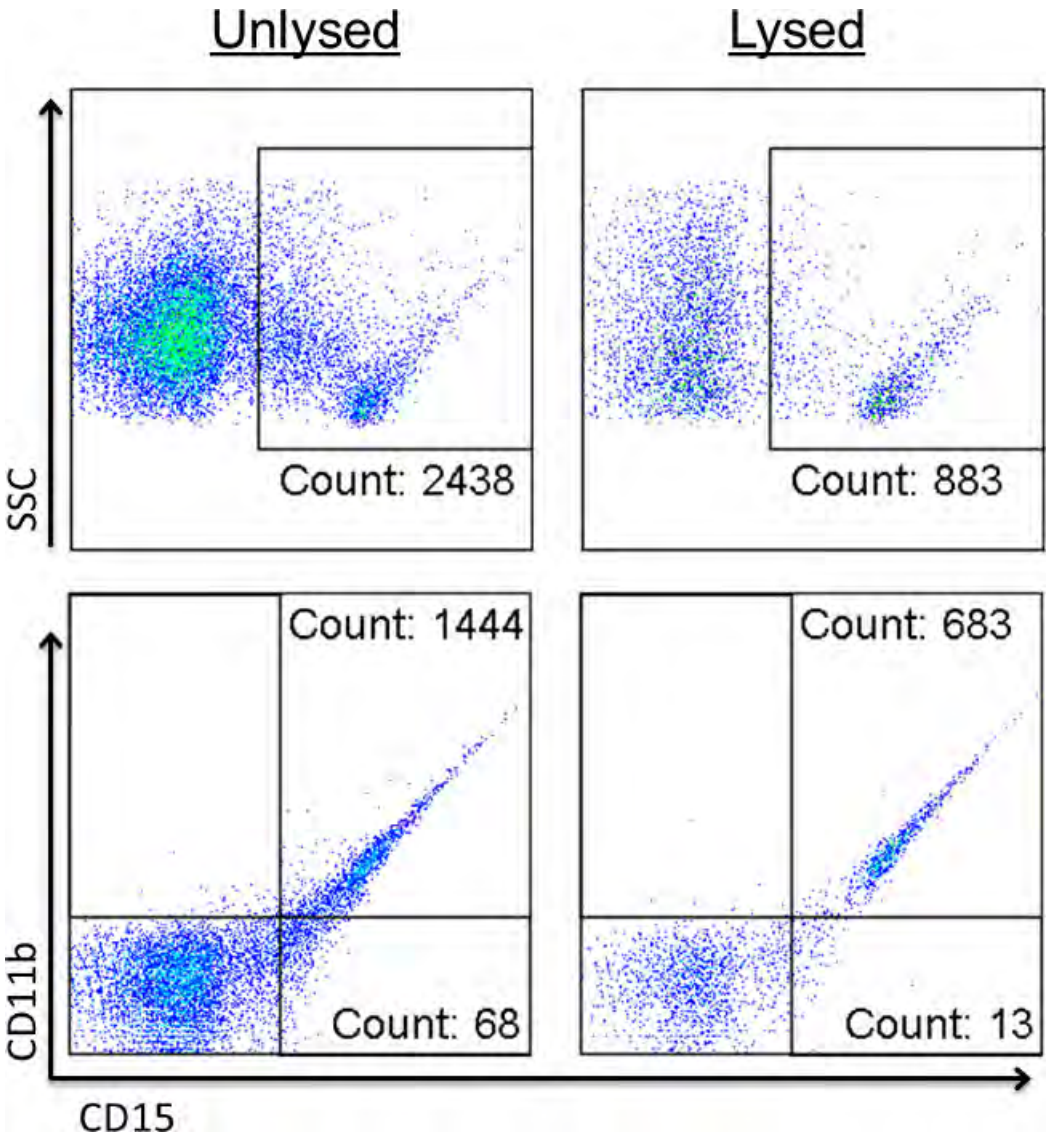
Figur 4 Compariso o expecte result usin bead vs individua detection Flo cytometr do plot sho representativ stainin of EV couple t bead (to row) compare t EV analyzin usin individua detectio (botto row) Event show i th righ biparamete plots ar withi th FSC/SS bead gate o th left.



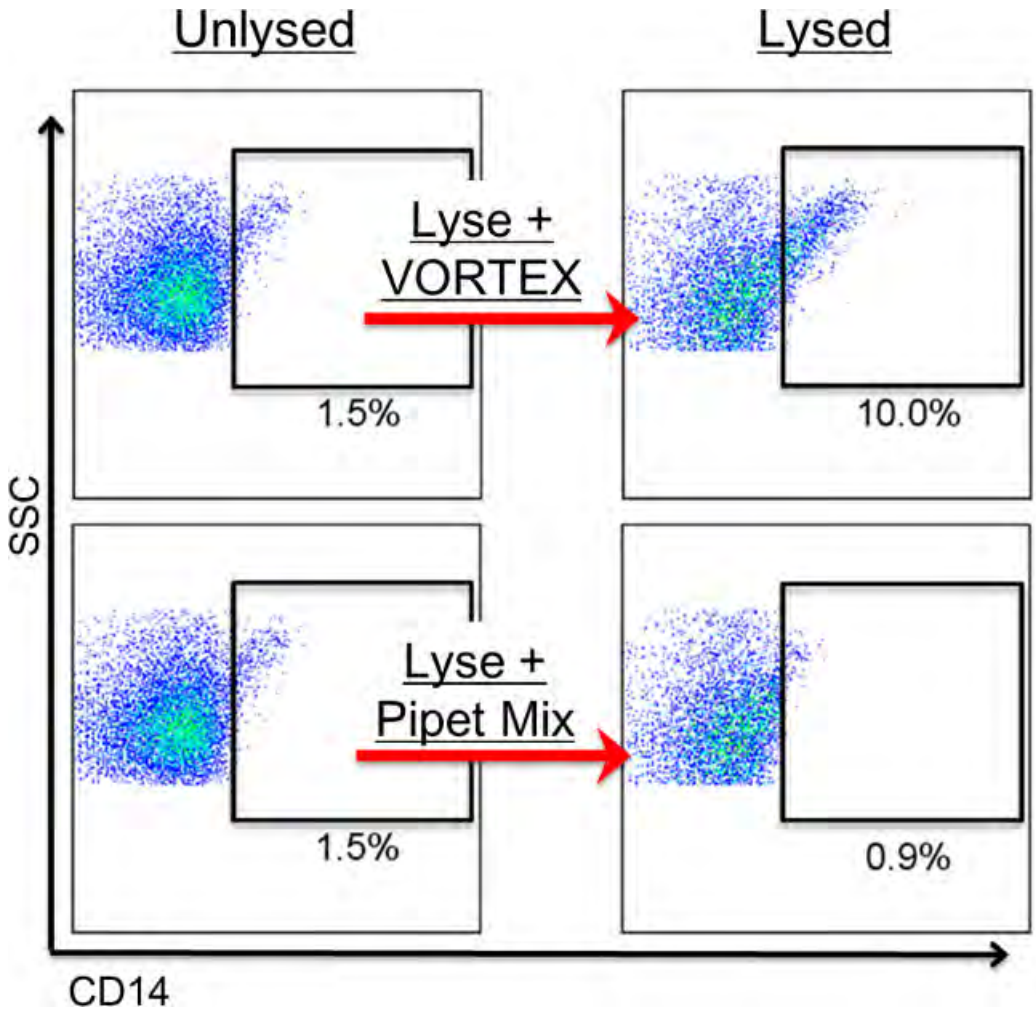
Figu e : Comparis n f differe t negati e contro s n individu l detecti n analysi . Valu s sh w percentag s f positi e event . Events sho n a e with n t e FSC/S C V gat . **( )** Comparis n f negati e contro s n unwash d v . wash d sample . Isoty e r lys d contro s were evaluat d f r the r abili y o provi e appropria e indicatio s f backgrou d fluorescen e acro s t o differe t marke s n a ful y stain d sample (bott m row . Gat s f r ea h mark r we e ma e usi g t e lys d samp e (t p ro) a d th n copi d o t e ro s beneat . Wash d sampl s were wash d usi g post-sta n filtratio . **( )** Examp e f a samp e stain d wi h t e isoty e contr l havi g mo e positi e even s th n t e samp e stained wi h actu l antibody.



Figur 6 Effec o post-stai washin i individua detection Value sho percentage an number o positiv events Event show are withi th FSC/SS E gate Gate fo eac sampl wer mad usin eac sample' lyse counterpar (no shown refe t previou figur for gate-settin i unwashe v washe samples) Hig backgroun fluorescenc make distinguishin positiv fro negativ event difficul (top plot) Whe washed however th positiv populatio i reveale a unboun fluorescen antibodie ar remove an backgroun fluorescence i reduce (botto plot).



Figur 7 Detergen lysi confirm th presenc o non-E signals Event show ar withi th FSC/SC E gate Value show percentage o positiv events Staine E sample wer rea befor (lef column an afte additio o detergen (righ column t identify positiv signal cause b immun complexe an othe non-EV-relate events.



Figur 8 Vortexin cause th appearanc o non-E signals Event show ar withi th FSC/SC E gate Value sho percentage of positiv events Staine E sample wer rea befor (lef column an afte additio o detergen (righ column) Usin vorte t mi samples ca caus EV-mimicking diagona population t for (to row) Whe mixe gentl u an dow usin pipet however formatio o these population ca b avoide (botto row) Vortexin i no recommende fo mixing a i ca caus aggregatio i som samples leadin to diagonal-appearing positiv populations I general even numbe withi positiv gat shoul no increas afte lysing.

	Bead-Base Detection	**Individua Detection**
E Sizes	recommende fo 10 n only	recommende fo 10 n only
Time	require overnigh incubation	ca complet i day
Sensitivity	cluste detection	singl particl detection
Results	qualitative	quantitative
Washing	simple standar centrifugation	require centrifuga filters
Negativ control	BSA-coate beads	lyse samples

Tabl 1 Pro an con o bot detectio methods.

Discussion

Tw differen protocol fo th isolation treatmen an analysi o EV wer presented usin eithe a individua detectio o bead-based approach Selectin th mos appropriat metho t us i no alway straightforwar an require a understandin o th sampl bein tested a wel a th individua subpopulation o interest Furthermore th sensitivit o th cytomete use fo acquisitio mus b considere when choosin th mos appropriat method Oftentime ther i n singl bes protoco t use rather combinatio o method provide more informatio abou sampl tha an on metho alone Ideally severa differen isolatio an detectio technique shoul b evaluate first i orde t develo tailore protoco tha take int consideratio individua cytomete performanc wit respec t th specifi E population bein studied Alternativ isolatio technique includ ultracentrifugation sucros densit fractionation immunomagneti bea separation, chromatography an affinit purification[12] whil alternativ detectio method includ scannin electro microscopy transmissio electron

microscopy, atomic force microscopy, dynamic light scattering, and western blotting[8]. By combining different techniques, the methods presented here can be adapted in order to create protocols best suited for studying various EV populations of interest.

In general, individual detection of EVs using FCM works well for analyzing larger EVs but loses sensitivity as EVs get smaller. While individual detection is more consistent in detecting larger EVs, bead-based detection is less sensitive in detecting larger EVs and more sensitive for exosomes. Larger EVs can be washed easily via post-stain filtration and detected singly via FCM. Smaller EVs and exosomes, on the other hand, are not detected well individually using FCM and are much more difficult to wash post-staining. The bead-capture protocol resolves both of these issues, allowing EVs to be easily washed and multiple EVs to be measured together to create larger positive signals detectable by FCM. However, there are drawbacks associated with using this method, as outlined in **Table 1**.

When working with a less sensitive cytometer, the capacity for individual detection is limited. Prior to EV analysis, the sensitivity of the cytometer should be determined using a mixture of bead sizes ranging from 0.1-1.0 µm. Failure of the cytometer to detect a majority of particles below 1.0 µm would necessitate the use of the bead-based protocol. Highly expressed markers are easily detected using either protocol. Rarer populations are sometimes easier to detect using the single particle detection protocol rather than the bead capture protocol, however, this can vary depending on such variables as: the brightness of the fluorochrome, the sample's EV:bead ratio, and the size of the EV bearing the rare cell surface marker. Detection of multiple markers on a single particle necessitates the use of the individual detection method. The bead-based method is not capable of individual EV detection. Therefore, the bead-based protocol will yield data that are more qualitative in nature, while the individual detection method will give more quantitative data.

Additional isolation techniques must be utilized whenever EVs are needed for downstream applications. EVs used in functional assays should be ultracentrifuged using the 3-step differential centrifugation protocol, since the soluble serum proteins in plasma can affect functional experimental outcomes. For characterization of EVs, however, ultracentrifugation is not recommended, since this added step may affect EV quality and quantity due to the high forces imparted on the particles[12].

The individual detection protocol contains several key steps optimized for high-throughput testing, including: 1) the implementation of centrifugal filters for the quick and effective removal of positive events caused by Ab aggregates, 2) the use of filters as a more practical alternative to ultracentrifugation or sucrose gradient fractionation for washing unbound Ab from EV samples post-staining, and 3) utilization of detergent lysis as a negative control, which not only reveals positive events caused by non-EVs but provides a good approximation of background fluorescence to distinguish positive from negative populations for drawing gates. The individual detection protocol is recommended whenever a large number of samples needs testing as it can be performed in a single day, whereas the bead-based method requires an overnight incubation.

The negative controls in each protocol have different advantages and disadvantages depending on which detection method is used. One benefit of using the bead-based assay is that the same monoclonal antibodies can be used for negative and positive tubes and the same negative control can be used for all samples. The individual detection method, on the other hand, requires separate controls to be read for each sample tested. The negative control used by the individual detection protocol uses lysed samples, which do not require the use/consumption of additional ant bodies but do require that each tube be read a second time after addition of the lysing agent. The lysed controls have the added benefit of being able to identify the proportion of positive signal that can be attributed to non-vesicle-related events such as immune complexes [21]. The bead-based assay does not have this ability todistinguish between positive signals arising from true EVs and those arising from non-vesicles.

Limitations of the technique

While there is no standardized method for the isolation of EVs, differential centrifugation is a widely used technique among EV researchers. The differential centrifugation method descr bed here is based on common protocols for isolating PPP, which typically require an initial centrifugation between 1,200-1,500 x g for 10-20 min to remove cells, followed by a second centrifugation between 10,000-13,000 x g for 10-30 min to remove platelets [35]. The protocol described herein uses a centrifugation at 1,500 x g for 10 min followed by a centrifugation at 13,000 x g for 10 min. While higher forces of 25,000-100,000 x g are typically required to pellet EVs, some of the larger EVs may be removed with the differential centrifugation protocol we have presented.

Up to 90% of EVs detected by FCM are lost with one hour ultracentrifugation at 100,000 x g (data not shown). Longer centrifugation times should be considered, albeit cautiously, as this may adversely impact the sample's composition. If additional processing is needed for characterization studies, filtration can be performed after the 2-step centrifugation (before staining) to further fractionate samples based on particle size. Similar to ultra-centrifugation, filtration can result in a loss of up to 50% of positive marker events and up to 90% of total particles detected by FCM (data not shown). While the increase in signal-to-noise ratio is of obvious benefit, the loss of smaller EVs represents a significant limitation when considering any washing or isolation method.

Finally, the anticoagulant used (*e.g.*, heparin, ACD, ethylenediaminetetraacetic acid (EDTA), *etc.*) during blood collection may impact the quality and quantity of EV content. While ACD has proven to be a good and reliable anticoagulant for our studies, testing multiple solutions is recommended to ensure that the most suitable anticoagulant for the application is chosen. This is especially important when EVs will be used in downstream assays where the anticoagulant used can affect the outcome. For example, some anticoagulants (*e.g.*, EDTA and heparin) are known to interfere with PCR reactions while others (*e.g.*, theophylline, adenosine and dipyridamole) have been shown to inhibit EV release from platelets[12].

Methods for EV analysis, while considerably improved over the last decade, are still a work in progress. Ultimately, the best methods for analyzing EVs will depend on the research being conducted and tools available to the researcher.

Disclosures

The authors have no conflict of interest to disclose.

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